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# INHIBITION OF PIGMENT-CELL MIGRATION IN TRITURUS TOROSUS

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DURING the later embryonic and early larval stages of the urodele *Triturus*, melanoblasts, arising from the neural crest, migrate ventrally on the sides of the body and, as pigment forms, become visible in a definite pattern (Twitty, 1945). In *Triturus torosus*, as pigment formation renders the cells directly visible in the earlier larval stages, a dorsal band, consisting of numerous cells, appears on each side of the dorsal mid-line. Slightly later a relatively small number of the pigment cells constitute a more or less definite longitudinal cell series just dorsal to the border of the ventral yolk on each side of the body, posterior to the head region. Still others, almost always fewer in number, may remain scattered between this lateral series and the dorsal band. After an interval, in which little or no increase in number of these lateral pigment cells is evident, additional cells become visibly pigmented.

In discussion of the behavior and the probable activity of the pigment cells of *Triturus*, one question which arose was this: Could this migration of melanoblasts be more or less inhibited or retarded by external agents without greatly affecting the progress of development in other ways? The neural crest, from which the pigment cells arise, represents the high end of the dorsiventral gradient pattern, as that is made visible by oxidation-reduction indicators (Child, 1943, 1948), and is also more susceptible to action of various inhibiting agents, e.g., potassium cyanide, than are more ventral regions of the embryo (Bellamy, 1919, 1922; Child, 1941, p. 153). The migrating cells are believed to be highly active, at least before and during the period of their migration. Consequently, it was thought that it might be possible to decrease the numbers of cells migrating ventrally without greatly affecting development in general. With this possibility in mind, experiments were undertaken on *T. torosus* during the breeding seasons of 1948 and 1949. The work was made possible by the kindness of Dr. Twitty in making available abundant embryonic material from his stocks.

## MATERIAL AND METHODS

*Triturus torosus* was chosen as the most satisfactory material for the purpose because the number of cells which become visible in lateral regions is smaller and the cells are less frequently clumped in groups, making exact counts difficult or impossible, than in some other available species. Figure 1, provided by

the kindness of Dr. Twitty and to appear in a later paper by him is reproduced from a drawing of a photograph of a living *T. torosus* larva, showing the exact number and localization of pigment cells visible on the side of the body. The number of cells visible laterally in this individual is smaller than in many animals. Cells are scattered over the side of the head region and between the gills and the anal body-level and form a longitudinal series adjoining the yolk border, consisting of nine cells. At the base of the gills are two more cells at the same distance from the dorsal band as the series posterior to the gills, making a total of eleven. One or more cells may perhaps be hidden by the gill filaments, as is often the case. Between the gills and the anus there is only one cell partway down to the yolk, or, if two cells dorsal to the gills and one apparently partially hidden by the second gill filament are included, there are four cells partway down. Posterior to the anal level, several cells are visible. For cell counts the region between the gills and the anal level was chosen because in this region the cell numbers tend to be relatively small and less variable than on the sides of the head and tail and the cells are less frequently clumped in groups. Other figures of early pigment pattern in *T. torosus* appear in an earlier paper by Twitty (1945, Figs. 1, *a–c*).

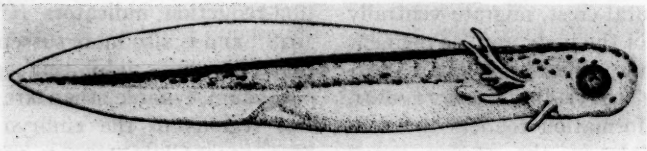

FIG. 1.—Visible pigment cells in an early larval stage of *Triturus torosus*, showing the longitudinal series of cells adjoining the yolk border, the A-cells of this paper, a few cells partway down to the yolk, the B-cells of this paper, and the dorsal bands.

Sodium azide was used as the inhibiting agent during the breeding season of 1948 and potassium cyanide during the season of 1949. A considerable part of each of these seasons was necessarily spent in preliminary experiments with different concentrations of the agents and with exposure at different developmental stages, in order to determine whether definite results could be obtained and, if so, what the effective ranges of concentrations, developmental stages to be exposed, and lengths of exposure periods were. In all cases experimental and control lots of a single experiment were taken from the same egg mass. Numbers of individuals in a particular lot varied with numbers in the egg mass and the number of lots into which it was divided. They usually ranged from four or five to ten, twelve, and occasionally thirteen. The smaller egg masses were usually divided into one lot subjected to the agent and one control in water, of equal, or almost equal, numbers of individuals. Larger egg masses were frequently divided into one control lot and two or even three lots in different concentrations, all approximately equal in numbers at the beginning of the experiment.

The embryos were removed from the jelly but remained in the membrane until they emerged in the natural way. This procedure was found to have some disadvantages. In the finger bowls used as containers the embryos within the membranes tended to slide over the surface and become aggregated in the deepest parts of the bowl in consequence of un-

avoidable slight jarring. If they were not frequently separated, embryos near the middle of the aggregation not infrequently died, both in controls and in experimental lots, presumably from lack of oxygen or accumulation of $CO_2$.

Even after the preliminary experiments showed that positive results could be obtained with certain ranges of concentration of the agents and without very high death rates, the experiments with azide were still to some extent exploratory, those with cyanide perhaps slightly less so. Concentrations of azide used in the data tabulated in this paper ranged from M/200 to M/500, and the stage at which exposure was begun varied from yolk plug through early and later neurulae to stages after closure of the neural folds. As will appear, the results with azide are definite but are not closely correlated with concentrations, apparently because of the considerable difference in developmental stages used in different lots. They suggested that, while exposure to the agent should be as brief as possible, it should preferably begin at the earlier stages used with azide. Consequently, in the tabulated experiments with cyanide in 1949, exposure was begun at stages ranging only from late yolk plug to early neurula with neural plate barely visible, or with earliest stages of neural folds. Concentrations of cyanide found to be most satisfactory ranged from M/13,333 to M/35,000. The azide material was kept in finger bowls covered by glass plates, with frequent agitation to spread aggregations and frequent change of solution; each lot was in 250–350 ml. of solution. The cyanide material was also kept in finger bowls similarly covered, but with very small or no air space and with frequent change of solutions. Deaths in azide and cyanide lots resulted in almost all cases from accumulation of liquid beneath the body wall in the ventral yolk region and final rupture of the body wall.

In a few individuals fluid accumulation appeared in the head region; this occurred only in the later stages of exposure to the agents. Concentrations were not high enough to produce differential disintegration. The animals remained in the azide or cyanide solutions until the cells were deeply pigmented and the larvae were 9–10 mm. in length, with gills and balancers and foreleg buds. General development was, of course, retarded in the experimental lots as compared with the controls; and in the higher concentrations sensitivity to mechanical stimulation was less and swimming movements were weaker than in controls, but no abnormalities in development were observed except a few slight indications of differential effect, to be mentioned later.

Completely accurate counts of cells adjoining the yolk were often possible, but occasionally some uncertainty resulted from clumping of cells, though the size of the mass was believed to permit an estimate of the number of cells concerned. As regards counts of cells partway down to the yolk, there was often difficulty in deciding whether a given cell should be regarded as adjoining the yolk or partway down or, in other cases, as part of the dorsal band. In the earlier azide lots, only the cells adjoining the yolk were counted; but, as the experiments progressed, it became evident that cells scattered between the dorsal band and those cells which had attained the yolk border should also be counted. Consequently, in all later azide lots and their controls and in all cyanide lots and controls, not only the cells adjoining the yolk border but also the cells partway down from the dorsal bands to the yolk border were counted and listed separately, and the sum of their numbers was listed as total for the individual. The re-

sults indicate that the distinction is of some value. Numbers of cells on the two sides of the body of an individual usually do not differ very greatly and are often almost identical. In different individuals from the same egg mass, the variation in number is greater; and in different individuals from different egg masses it is still greater. Also material from different localities may show considerable difference in cell number. For example, both Dr. Twitty and the writer have observed that material from a locality on the slope of Mount Hamilton, some fifty miles from Palo Alto, averages a higher number of cells in lateral regions than does material from localities immediately about Palo Alto; but other distinguishing differences have not been found. The data of this paper are necessarily based on material from different sources; variations in temperature during the experiments were slight, but the aggregations of individuals in the containers noted above may have inhibited cell migration to some extent in some animals. There is also less motor activity in azide and cyanide than in controls; and, after hatching, larvae may perhaps remain lying on one side long enough to inhibit cell migration to some extent on the side in contact.

Determination of the proper stage for cell counts presented some difficulties. Even in the same lot from a single egg mass, some individuals were more inhibited than others. Recounts on the same individuals, made in many cases 2–4 days after the first count, showed only slight differences in cell numbers. All recounts were made without knowledge of the first counts, and the animals were not in the same order in the two counts.

The chief results are tabulated as individual averages for all lots in a particular concentration of azide or cyanide and for the corresponding controls from the same egg mass in each lot. The azide and cyanide averages are also tabulated as percentages of the controls. Calculations of averages and controls were carried to two or three decimal places, and the single decimal in the tabulated data is the closest approach to the calculated value.

The use of material from different sources; the different developmental stages at the time of exposure to the agents, more particularly in the azide lots; the differences in susceptibility in individuals from the same, and from different, egg masses; and the possible effects of aggregation and of lying on one side are regarded as making any attempt at statistical treatment of the material of little or no real value. For the purpose of such treatment, material from a single locality should be used and should be subjected to the agent at a particular developmental stage, and the possible effects of aggregation and of lying on one side should be prevented as completely as possible, perhaps by leaving the animals in the jelly and by frequent agitation of the solutions after hatching.

### THE EFFECT OF SODIUM AZIDE

Both the material in azide and the controls were examined repeatedly in order to determine when the cells were sufficiently pigmented to permit counts. The time in azide ranged from 12 to 16 days except for one lot in M/200 for 18 days and one lot in M/300 for 22 days. Cell counts were made in controls 9–12 days after removal from the jelly. In general, the length of time in azide was greater with the higher concentrations.

The more important results obtained with azide appear in Table 1. Concentrations of azide are listed in the first column and the number of larvae in each concentration on which cell counts were made, in the second column. Deaths in azide or controls preceding cell counts are not

listed. Numbers of larvae on which counts were made are also listed in the first control column. The three columns headed "A," "B," and "AB" under azide and controls give the individual averages for azide concentrations and controls: the column A gives averages of cells adjoining the yolk border; B, of cells partway down to the yolk; and AB, the sum of A and B, i.e., all visible cells below the dorsal bands. In the earlier azide experiments, only the cells adjoining the yolk were counted. In M/200 and M/250 azide and in corresponding controls no other counts were made. For M/300, M/400, and M/500 two sets of averages appear, distinguished as A and AB. In the A-cases, only the A-cells were counted; in the AB-cases the B-cells were also counted and averages of A-, B-, and AB-cells appear. Under "Percentages" the averages of A-, B-, and AB-cells are given as percentages of the averages of corresponding cells in the controls. The table includes data for lots from 13 egg masses in the A-groups, a total of 31 animals in azide and 48 controls; and lots from 6 egg masses in the AB-groups, a total of 33 animals in azide and 31 controls.

The control averages of the A-lots show a range of variation from 13.7 to 39.5. In the AB-lots the range is from 23.5 to 30 for the A-cells, from 5.9 to 11.6 for the B-cells, and from 29.4 to 41.6 for AB, the totals. The range of variation is, in general, less in azide: in the A-lots it is from 4 to 9.7; in the AB-lots, from 10 to 13.4 for the A-cells, from 6.2 to 9 for the B-cells, and from 18.9 to 20.9 for AB, the totals. If more active cells are more susceptible to azide, less variation is to be expected in azide than in controls.

Turning to the azide averages as percentages of the control averages, the range for the A-lots is from 12.9 for M/200 to 65.5 for M/300 with percentages of 48.5 in M/400 and 32.9 in M/500. The decrease to 12.9 per cent of the control average in M/200 is of particular interest as indicating the effect of the highest concentration of azide found to be usable without a high death rate.

TABLE 1

INDIVIDUAL AVERAGES OF NUMBERS OF PIGMENT CELLS WHICH HAVE MIGRATED VENTRALLY IN DIFFERENT CONCENTRATIONS OF SODIUM AZIDE AND IN CONTROLS IN WATER AND AZIDE AVERAGES AS PERCENTAGES OF CONTROL AVERAGES

| Concentration | Azide | | | | Controls | | | | Percentages | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of Embryos | A | B | AB | No. of Embryos | A | B | AB | Azide A/ Control A | Azide B/ Control B | Azide AB/ Control AB |
| M/200 A | 6 | 4 | ..... | ..... | 12 | 30.9 | ..... | ..... | 12.9 | ..... | ..... |
| M/250 A | 2 | 9 | ..... | ..... | 2 | 39.5 | ..... | ..... | 22.8 | ..... | ..... |
| M/300 A | 7 | 9 | ..... | ..... | 8 | 13.7 | ..... | ..... | 65.5 | ..... | ..... |
| M/300 AB | 8 | 10 | 9 | 19 | 14 | 23.5 | 5.9 | 29.4 | 42.6 | 153.7 | 64.8 |
| M/400 A | 16 | 9.7 | ..... | ..... | 15 | 20 | ..... | ..... | 48.5 | ..... | ..... |
| M/400 AB | 10 | 13.4 | 7.5 | 20.9 | 12 | 30 | 11.6 | 41.6 | 44.7 | 64.6 | 50.2 |
| M/500 A | 11 | 7.4 | ..... | ..... | 11 | 22.5 | ..... | ..... | 32.9 | ..... | ..... |
| M/500 AB | 4 | 12.7 | 6.2 | 18.9 | 5 | 29 | 11.2 | 40.2 | 43.8 | 55.3 | 47 |
| All concentrations: A | 31 | 7.8 | ..... | ..... | 48 | 25.3 | ..... | ..... | 30.8 | ..... | ..... |
| AB | 33 | 12.3 | 7.6 | 19.9 | 31 | 27.5 | 9.6 | 37.1 | 44.7 | 79.2 | 53.6 |

The high percentage, 65.5, for the A-lot in M/300 is probably due to the fact that 3 of the 9 individuals in this lot were exposed to azide only after closure of the neural folds and 4 individuals at the earliest stages of gill buds. At these stages migration of the pigment cells is undoubtedly advanced. However, the decrease in percentages from M/300 to M/500, i.e., with decrease in concentration, cannot be accounted for in this way and is opposed to expectation.

In the AB-lots in M/300, M/400, and M/500 the percentages for the A-cells—42.6, 44.7, and 43.8—do not differ greatly, but those for the B-cells—153.7 in M/300, 64.6 in M/400, 55.3 for M/500—show much greater difference and also decrease with decrease in concentrations. The percentages for the AB-cells also decrease somewhat with decrease in concentration, but the decreases are less than in the A-lots. One point of interest concerning the B-cells which does not appear in Table 1 is that they constitute higher percentages of the AB-totals in azide than in controls. In M/300 they are 47.8 per cent of the AB-cells, in the corresponding controls 20 per cent; in M/400 they are 35.8 per cent of the AB-cells as compared with 27.9 per cent in controls; in M/500 they are 32.9 per cent of AB-cells as compared with 27.8 per cent in controls.

As regards the decrease in percentages for the AB-cells from 64.8 in M/300 to 47 in M/500, it may be noted that most, if not all, of this decrease in percentages is due to increase in the averages for the AB-cells of the controls from 29.4 to 41.6 and 40.2. Averages for the AB-cells in these concentrations—19, 20.9, and 18.9—differ by only two cells. The AB-lots of Table 1 were from material obtained later in the season than the A-lots.

The point of chief importance in Table 1 is that in the data for all concentrations the A-cells adjoining the yolk border in the A-lots and the A-cells and AB-cells of the AB-lots average much less in number than those in the controls. In all individuals from 18 of the 19 egg masses included in Table 1, the A- and AB-cells were distinctly fewer than in the corresponding controls. In material from a single egg mass the individual average in azide M/400 was only slightly less than in the control. The B-cells in azide M/300 average more than in the control and less in M/400 and M/500, but the differences are only 3–5 cells. In spite of the uncertainties, it seems beyond question that the numbers of pigment cells which become visible ventral to the dorsal bands are very considerably decreased by subjection to sodium azide in concentrations of from M/200 to M/500.

The general averages and percentages for all concentrations together in the last two lines of Table 1 serve merely to indicate in some measure the general effect of azide in decreasing numbers of visible pigment cells. For the A-cells of the A-lots the general average in azide is 30.8 per cent of the control average. In the AB-lots the A-cells are 44.7 per cent, the B-cells 79.2 per cent, and the AB-cells 53.6 per cent of the control averages. The lower percentage of the A-cells of the A-lots, 30.8, than for the A-cells of the AB-lots, 44.7, results from the great decreases in average numbers in M/200 and M/250. The AB-lots were not subjected to these concentrations. The general percentage, 53.6, for the AB-cells, i.e., for all cells below the dorsal bands, indicates a decrease in number of visible cells by azide of almost 50 per cent.

### THE EFFECT OF POTASSIUM CYANIDE

Table 2 is like Table 1 as regards manner of presentation of data. It includes individual averages for the concentrations of cyanide used, and the cyanide

averages are also tabulated as percentages of control averages. The table consists of material in cyanide and control from 20 egg masses. The animals in M/13,333 and M/20,000 were from the same egg mass, and the same control was used for both, as the table shows. Consequently, this control was used only once in determining the total number of control animals included in the table. The number of animals in cyanide was 130, the number of controls 123. Both A- and B-cells were counted in all cases, and lots. The B-cells partway down are more numerous than in controls, and the percentages are consequently above 100 in four of the six lots and above 250 in M/16,000. One of the preliminary experiments with cyanide, concerned with determining the lower effective limit of concentration, is not included in Table 2 because only the A-cells were counted, but it is of some interest in connection with the table. It consisted of 5 animals in cyanide M/50,000 and 5 controls from the same egg mass. The individual aver-

TABLE 2

INDIVIDUAL AVERAGES OF NUMBERS OF PIGMENT CELLS WHICH HAVE MIGRATED VENTRALLY IN DIFFERENT CONCENTRATIONS OF POTASSIUM CYANIDE AND IN CONTROLS IN WATER AND CYANIDE AVERAGES AS PERCENTAGES OF CONTROL AVERAGES

| Concentration | Cyanide | | | | Controls | | | | Percentages | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of Embryos | A | B | AB | No. of Embryos | A | B | AB | Cyanide A/ Control A | Cyanide B/ Control B | Cyanide AB/ Control AB |
| M/13,333 | 6 | 9.3 | 2.3 | 11.6 | 5 | 20.8 | 8.4 | 29.2 | 44.8 | 27.4 | 39.8 |
| M/16,000 | 5 | 13.6 | 8 | 21.6 | 11 | 59.9 | 3.1 | 63 | 22.7 | 258.1 | 34.3 |
| M/20,000 | 6 | 12.5 | 4.7 | 17.2 | 5 | 20.8 | 8.4 | 29.2 | 60 | 55.5 | 58.9 |
| M/25,000 | 56 | 17.2 | 6 | 23.2 | 40 | 32.6 | 3.5 | 36.1 | 52.8 | 174.3 | 64.5 |
| M/30,000 | 37 | 20.5 | 4.7 | 25.2 | 40 | 28.6 | 4.4 | 33 | 71 | 106.8 | 75.7 |
| M/35,000 | 20 | 16.5 | 4.8 | 21.3 | 22 | 21.8 | 4.4 | 26.2 | 75.7 | 109.1 | 81.3 |
| All concentrations | 130 | 14.9 | 5.1 | 20 | 123 | 32.7 | 4.8 | 37.5 | 41.3 | 106.4 | 53.3 |

their averages and the AB-averages for total cells appear for all lots in the table. Animals were in cyanide 11–19 days preceding cell counts, except for one case in cyanide 25 days and one 22 days preceding counts, both of these in the higher concentrations. Cell counts were made on controls 10–13 days after removal from the jelly.

Except for the slightly higher percentage, 39.8, for the AB-cells in M/13,333 than 34.3 for the AB-cells in M/16,000, the percentages for these cells increase with decreasing cyanide concentration to 81.3 in M/35,000. The percentages for the A-cells increase with decreasing concentration of cyanide in four of the six age for cyanide was 46.8 and for controls 55.8; and the percentage was 83.9, still higher for this lower concentration than the percentage 75.7 for the A-cells in cyanide M/35,000.

The last line of Table 2 gives total numbers of individuals counted in cyanide of all concentrations and in controls and general individual averages and percentages for all lots in cyanide and all controls. These general data are compared with those of Table 1 in the following section. Here only one point, not tabulated but of some interest, need be noted: the B-cells are 25.5 per cent of the AB-totals in cyanide and 12.8 per cent in the controls. In other words, the cells

partway to the yolk border constitute in cyanide a percentage of total cells twice that in the controls.

### COMPARISON OF THE EFFECTS OF AZIDE AND CYANIDE

As regards certain points, a comparison of Tables 1 and 2 appears of interest. The individual averages and percentages for the different concentrations of azide and cyanide provide no adequate basis for comparison beyond the evident decrease in both agents as compared with controls. However, the general averages and percentages in the last two lines of Table 1 and the last line of Table 2 show certain characteristics which appear to justify a brief comparison.

The general averages for the AB-lots of Table 1 in azide—12.3, 7.6, and 19.9—and for all cyanide concentrations in Table 2—14.9, 5.1, and 20—differ very little. The general average for the A-lots of Table 1—7.8—is below the 12.5 for the A-cells of the AB-lots, largely because of the small numbers of these cells in azide M/200 and M/25c. The percentages for the A-cells of the AB-lots of Table 1—44.7—and for the A-cells of Table 2—41.3—also show little difference, and the percentages for the AB-totals—53.6 in Table 1 and 53.3 in Table 2—are almost identical. The general percentage for the A-lots of Table 1—30.8—is considerably lower, again because of the great decrease in numbers of the cells in the highest azide concentrations, which were not used on AB-lots.

The B-cells are few in number and their general averages from the AB-lots of Table 1 differ in azide and controls by only 2 and in cyanide and control in Table 2 by only 0.3. These cells are more numerous in azide than in control in only one lot of three, in cyanide in four lots out of six. The percentages—79.2 for azide and 106.4 for cyanide—differ by 27.2. This difference suggests that a larger proportion of the migrating cells becomes visible partway down to the yolk border in cyanide than in azide, although the averages and percentages for all cells migrating are almost identical in azide and cyanide. When it is recalled that the data on cyanide were obtained a year later than those on azide and without regard to the localities from which the material was collected and that the data were not compared until the two tables were completed, the very close similarity of the general averages and percentages seems rather remarkable.

### INDIVIDUAL CELL COUNTS

Tables 1 and 2 give only individual averages determined from the records of individual cell counts. The tabulation of any considerable number of cell counts is out of the question, but an example of the counts as made on all individuals is presented in Table 3. This series of individuals in cyanide and controls—of course, from the same egg mass, as in all other single experiments—was chosen for presentation because it included a larger number of individuals than most cases from single egg masses and because only one death occurred, a control. Comparison with the data of other experiments indicates that it is fairly characteristic of the differences in cell numbers on the two sides of the body and in different individuals from the same egg mass. The table gives the individual counts for A- and B-cells on right and left sides of the body in 13 animals in cyanide M/25,000 and 12 controls in water, the total numbers of these cells, and the individual averages for right and left sides. Below the table individual averages for A-, B-, and AB-cells and the percentages, as in Tables 1 and 2, are given. The cyanide average for the A-cells is somewhat lower, that for the B-cells higher, and that for the AB-

cells slightly lower, than in the 56 animals of Table 2 in cyanide M/25,000. The control averages are almost the same as in Table 2. The percentage for the A-cells, 30.4, is slightly more than half of the 52.8 for A-cells in the same cyanide concentration of Table 2; that for the B-cells, 239, is 64.7 above the 174.3 of Table 2; and the percentage for the AB-cells, 53.2, is less than the 64.5 of Table 2. Though not stated in connection with Table 3, it may also be noted that the B-cells are 49 per cent of the AB-cells in cyanide and only 9.2 per cent of the AB-cells in the controls.

In the cyanide animals of Table 3, numbers of A-cells differ by more than 3 on the two sides of the individual body in

TABLE 3

INDIVIDUAL CELL COUNTS ON RIGHT AND LEFT SIDES IN POTASSIUM CYANIDE M/25,000 AND IN CONTROLS IN WATER

| Cyanide (13 Animals) | | | | Controls (12 Animals) | | | |
|---|---|---|---|---|---|---|---|
| Right | | Left | | Right | | Left | |
| A | B | A | B | A | B | A | B |
| 10 | 9 | 6 | 3 | 17 | 0 | 20 | 0 |
| 6 | 5 | 4 | 4 | 14 | 1 | 15 | 1 |
| 8 | 6 | 8 | 8 | 16 | 1 | 17 | 0 |
| 1 | 8 | 1 | 4 | 19 | 3 | 20 | 2 |
| 6 | 6 | 6 | 5 | 12 | 1 | 16 | 2 |
| 4 | 5 | 2 | 8 | 14 | 4 | 20 | 3 |
| 5 | 3 | 8 | 0 | 16 | 3 | 8 | 0 |
| 7 | 5 | 5 | 6 | 16 | 4 | 15 | 0 |
| 6 | 4 | 7 | 3 | 17 | 0 | 17 | 2 |
| 3 | 2 | 0 | 2 | 22 | 0 | 14 | 3 |
| 3 | 4 | 5 | 5 | 21 | 0 | 24 | 0 |
| 3 | 6 | 2 | 5 | 19 | 1 | 13 | 2 |
| 6 | 7 | 11 | 4 | ..... | ..... | ..... | ..... |
| 68 | 70 | 65 | 57 | 203 | 18 | 199 | 15 |
| 5.2 | 5.4 | 5 | 4.4 | 16.9 | 1.5 | 16.6 | 2.6 |

INDIVIDUAL AVERAGES, BOTH SIDES

| Cyanide | | | Controls | | |
|---|---|---|---|---|---|
| A | B | AB | A | B | AB |
| 10.2 | 9.8 | 20 | 33.5 | 4.1 | 37.6 |

PERCENTAGES

| Cyanide A/ Control A | Cyanide B/ Control B | Cyanide AB/ Control AB |
|---|---|---|
| 30.4 | 239 | 53.2 |

only 2 animals; in the controls, in 5 animals. Numbers of B-cells on the two sides of the body differ by more than 2 in 5 animals in cyanide and in 5 controls. The total numbers of A-cells on right and left sides in cyanide, 68 and 65, differ by only 3 cells; and in the controls, 203 and 199, by only 4 cells. The total numbers of B-cells on the two sides differ by 13 cells in cyanide and by only 3 in controls. The large difference in cyanide may be an effect of lying on one side. This was noted as a possible complicating factor in the section on "Material and Methods."

The variations in different individuals of Table 3 are much greater than on the two sides of the individual body. In cyanide they range from 1 to 10 on the right side and from 1 to 11 on the left for the A-cells; from 2 to 9 on the right and from 0 to 8 on the left for the B-cells. In the controls they range from 12 to 22 on the right and from 8 to 24 on the left for the A-cells; for the B-cells they range from 0 to 4 on the right and from 0 to 3 on the left.

### RECOUNTS

Recounts were made in almost one-third of the single experiments, the second counts 2–4 days after the first, in order to determine whether the first counts were fairly accurate and whether there was any considerable increase in numbers of cells in the interval between counts. In all cases the second counts were made without knowledge of the first, and individuals were not counted in the same order. Table 4 gives five examples of individual averages for first counts and recounts on the same individuals. The headings of the first and second columns of the table need no explanation. The third column gives the length of exposure to the agent or in water in days at the time of first and second counts. Under "A," "B," and "AB" individual averages are given as in Tables 1 and 2.

TABLE 4

INDIVIDUAL AVERAGES OF RECOUNTS OF PIGMENT CELLS

| Experimental Conditions | No. of Embryos | Experimental Period (Days) | Averages | | |
|---|---|---|---|---|---|
| | | | A | B | AB |
| Azide | 4 | 13 | 14.5 | 9 | 23.5 |
| M/400 | | 15 | 16 | 5.2 | 21.2 |
| Cyanide | 8 | 15 | 16.2 | 0.9 | 17.1 |
| M/30,000 | | 18 | 18 | 2.1 | 20.1 |
| Cyanide | 8 | 16 | 15.6 | 9.1 | 24.7 |
| M/35,000 | | 18 | 18 | 8.8 | 26.8 |
| Cyanide | 5 | 21 | 11 | 6 | 17 |
| M/35,000 | | 25 | 11 | 4.1 | 15.1 |
| Control | 11 | 11 | 20.8 | 2.7 | 23 5 |
| Water | | 13 | 22.7 | 2.3 | 25 |

In the first case in azide with 2 days between counts, the differences between the two counts show a decrease of 2.3 in the AB-averages, i.e., no increase in total cells. In the three following cases in cyanide the differences are slight and do not indicate any marked increase in cell numbers. The fifth case, a control in water, also shows only slight differences. Factors concerned in the slight differences

between the two counts are the difficulty in deciding whether to regard certain cells as partway to the yolk border and therefore to be listed under B, uncertainty as to number of cells in occasional aggregations, and the possibility of occasional further migration of B-cells in the interval between counts so that in the second count they are listed as A-cells. As regards this last possibility, it may be noted that the A-cell averages range from 1.5 to 2.4 higher in the second count in four of the five cases in Table 4, while the B-cell averages decrease from first to second counts. It is evident from the table that no considerable change in cell numbers has occurred in the intervals between counts. Consequently, the counts can be regarded as fairly accurate.

TABLE 5

INDIVIDUAL AVERAGES OF PIGMENT-CELL COUNTS BEFORE AND AFTER RETURN TO WATER

| Experimental Conditions | No. of Embryos | Experimental Period (Days) | A | B | AB |
|---|---|---|---|---|---|
| Cyanide, M/25,000..... | 10 | 19 | 21.9 | 4.7 | 26.6 |
| Water..... | 7 | 8 | 18.8 | 3.4 | 22.2 |
| Cyanide, M/25,000..... | 8 | 18 | 20.8 | 7.1 | 27.9 |
| Water..... | 7 | 8 | 22.3 | 4.6 | 26.9 |

## RECOVERY

In a few cases animals from a single egg mass were brought from cyanide to water after first counts, and recounts were made after several days. Table 5 shows individual averages for two characteristic cases, the second counts being made after 8 days in water. In all except the A-cells of the second case the averages are lower in the second than in the first count. If the differences have any significance, they probably indicate a slight increase in aggregation, with the resulting uncertainty as to numbers of cells concerned. Here, as in Table 4, the first counts were fairly accurate, and there has been no increase in total numbers of cells. With longer recovery periods, increases in cell numbers did, of course, occur sooner or later and became rapid.

## INCIDENTAL OBSERVATIONS

Certain observations suggest a differential in susceptibility or sensitivity to the inhibiting agents at different levels of the longitudinal axis and its gradient pattern. In control animals pigment cells are scattered over the sides of the head region anterior to the gills, as shown in Figure 1, and in many individuals in considerably larger numbers than in the figure. These cells have not been included in the counts; but, when counts were made in azide or cyanide, particularly in the higher concentrations, it was noted that the numbers of pigment cells on lateral head regions were usually much less than in controls. They may be completely absent, although cells are present posterior to the gills; but even there they are often less numerous anteriorly than posteriorly or are present only in the posterior half, more or less of the preanal region and in small numbers, e.g., 2–5. In such animals the dorsal bands may be almost absent in the head region and contain fewer cells for a short distance posterior to it. In the

tail region the pigment cells are apparently less sensitive to the agents, and their numbers are considerably larger below the dorsal bands than in the preanal body. Animals returned to water for recovery after cell counts show some evidence of differential recovery of pigment cells. An increase in numbers of pigment cells in the head region earlier than in the lateral body regions has frequently been observed.

In the higher concentrations of the agents some cases of slight dorsal curvature of the body occurred. These apparently indicate slightly more inhibition of growth dorsally than elsewhere, i.e., a greater sensitivity to the agents at the higher levels of the dorsiventral component of the gradient pattern.

Pigmentation of the eye progresses posteroventrally from the anterodorsal region under natural conditions (Child, 1948, p. 92). In the higher concentrations of the inhibiting agents, pigmentation in some animals occurs at the same time all around the eye, and in a few individuals it has been observed to progress from the ventral or posteroventral region dorsally or anterodorsally, i.e., in a direction almost, or quite, the reverse of the normal This is obviously a differential inhibition related to the gradient pattern. The higher end of the gradient in the developing eye is anterodorsal; the lower end, posteroventral. The agents inhibit the pigment cells differentially to such an extent that the usual gradient is obliterated or reversed.

## DISCUSSION

It is evident that the numbers of pigment cells visible on the sides of the larval body of *T. torosus* can be very greatly decreased by sodium azide and potassium cyanide without producing other alterations in development except general retardation and certain slight indications of differential retardation, mentioned above. The range of variation in the individual averages and percentages, according to the concentration of the agents and apparently also to the sensitivity of the material, is great. In the highest concentration of azide the cells adjoining the yolk border are 12.9 per cent of the controls; in the lowest cyanide concentration they are 75.7 per cent of controls; in other concentrations of the two agents they are between these extremes. The general averages for all concentrations in Tables 1 and 2 differ very little and suggest that the two agents, in the range of concentrations found to be effective without too great a death rate, act in much the same manner on the pigment cells. However, the B-cells, the cells between the dorsal band and the cell series at the yolk border, though relatively few, vary more widely in number than do the other cells; and their individual average numbers in cyanide are greater than in controls more frequently than is the case in azide. In view of the heterogeneity of the material and the difficulty in deciding whether certain cells are, or are not, to be regarded as belonging to this group, it is perhaps questionable whether this difference has any actual significance.

The appearance of pigment is, of course, retarded in azide and cyanide, as compared with the controls; but the order of its appearance in the dorsal bands and the lateral regions has not been observed to differ greatly in the agents and in controls. It has been shown by DuShane (1939) and by Twitty and Bodenstein (1944) that there is a dorsiventral differential or gradient as regards developmental stage and rate of pigment development. Pigment develops earliest and most rapidly dorsally and progressively later and less rapidly at lower levels of the general dorsiventral gradient of the *Triturus* body. Apparently, the dorsi-

ventral gradient of pigment formation is related in some way to the general dorsiventral gradient. Grafting experiments also show that there is a definite correlation between the dorsiventral differential in rate of pigment development and the gradient level of tissues about, or, more strictly speaking, beneath, the pigment cells. The tentative suggestion is perhaps justified that the more active tissues of more dorsal levels of the general gradient provide more material for melanin formation.

According to Twitty (1945), the accumulation of pigment cells in the dorsal bands results from reaggregation of cells scattered from the dorsal region ventrally over the sides of the body. The mechanism of this reaggregation is believed to be retraction of interconnecting processes as the cells differentiate. Since differentiation of the cells is more rapid dorsally than at other dorsiventral levels, aggregation begins there. Some cells, apparently not at all, or only slightly, involved in the reaggregation, remain at the yolk border or between it and the dorsal band and constitute the A- and B-cells of Tables 1 and 2 of the present paper. In this connection a question arises concerning the effect of azide and cyanide on reaggregation. If reaggregation is decreased in degree or in spatial extent of effectiveness, it might be expected that more cells would be left ventral to the dorsal bands and would become visible as A- and B-cells as pigment developed. Actually, however, the cells below the dorsal bands which become pigmented in azide and cyanide are fewer than in controls, though the cells partway to the yolk border (B-cells) appear to be somewhat more numerous in cyanide than in controls. Reaggregation of the cells is evidently retarded by the agents, as is development generally; and, since the cells at the yolk border are apparently not involved in the reaggregation, it seems necessary to conclude either that fewer cells reach the yolk border in the agents than in the controls or that development of pigment in some of the cells which do reach the border is delayed much more than in those which do become visible. The cells partway to the yolk border (B-cells) constitute higher percentages of the total numbers of cells below the dorsal bands in azide and cyanide than in controls.

The evidence bearing on the two possible interpretations of the cell counts requires consideration. Do azide and cyanide decrease the migratory activity of the melanoblasts so that fewer cells reach the yolk border and relatively more cells remain partway to the border in the agents than in the controls, or do the agents merely retard or prevent pigment development in some of the cells which have migrated but fail to become visible because of the absence of pigment?

First, it is to be noted that cell counts on animals in azide and cyanide were made only when cells on the sides of the body had become as deeply pigmented as the cells in controls at the times when they were counted. Second, the dorsal bands appeared to be as deeply pigmented in the agents as in the controls when counts were made. Third, cells only slightly pigmented were not frequently observed in the agents. Fourth, the cells at the yolk border are all at approximately the same level of the general dorsiventral gradient pattern; if this is an important factor in determining developmental stage and rate at which pigment develops, it is to be expected that development of pigment in the cells at that dorsiventral body-level would occur at about the same time rather than at very different times. Fifth, the visible cells partway to the yolk border constitute higher percentages of total visible cells

below the dorsal bands, i.e., their numbers are relatively larger, in the agents than in controls. This difference suggests that they may be cells which have failed to reach the yolk border rather than merely part of the cells which have migrated to these levels and have become visible in consequence of pigment development, while other cells at the same levels remain invisible because their pigment development has been inhibited. Sixth, recounts in the agents 2–4 days after the first counts show no marked increases in numbers of visible cells and often a slight decrease, probably resulting, at least in part, from local aggregations. Seventh, no marked increases in numbers of visible cells have been observed after 8 days in water, following cyanide.

These lines of evidence are regarded as favoring the view that the differences in numbers of visible pigment cells below the dorsal bands in the agents and in controls, as regards the regions where counts were made, result, at least in part, from more or less inhibition or retardation of ventral migration of melanoblasts rather than merely from retardation or prevention of pigment development in some of the cells which have already migrated. However, the possibility that pigment development may be delayed or inhibited in some of the cells which have migrated is not excluded. Whether this is the case or not, it appears beyond question that the pattern of pigmentation in early larval stages is altered in a definite manner by the agents, with little effect on development in general except retardation.

If ventral migration of the melanoblasts is more or less inhibited by the action of azide and cyanide, it appears evident that the migratory activity of the cells is highly susceptible to the action of the agents. According to the evidence from differential susceptibility and from intracellular oxidation and reduction of indicators, these cells, derived from the neural crest, originate from the extreme high end of the dorsiventral gradient (Bellamy, 1919, 1922; Child, 1943, 1948) and might therefore be expected to be more susceptible than other cells ventral to them at the same anteroposterior body-level, particularly during their migratory period. The great decreases in numbers of visible cells in the agents is in agreement with this earlier evidence.

Some incidental observations concerning differentials in appearance of pigment cells along the anteroposterior axes of individuals under the inhibiting conditions were noted in the preceding section as suggesting quantitative differential susceptibility in relation to the longitudinal gradient pattern. If these observations are indicative of differential susceptibility, they suggest that the pigment cells originating at the higher, more anterior, levels of the longitudinal gradient pattern are more susceptible to inhibition than are those from the more posterior, lower, levels and also recover more rapidly after return to water, if the inhibiting action was not too extreme. Many other data on development and reconstitution in invertebrates and vertebrates indicate similar relations between gradient pattern and susceptibility to many external agents (Child, 1941, chaps. iv–vii).

## SUMMARY

1. As determined by individual counts on 64 individuals in sodium azide and 79 controls, 130 individuals in cyanide and 123 controls, the numbers of melanoblasts which migrate ventrally from the neural crest in *T. torosus* and become visible on the sides of the body between the base of the gills and the anal level in consequence of pigment formation are decreased by exposure to sodium azide and potassium cyanide from develop-

mental stages before the migration has begun or before it has progressed far.

2. Individual averages vary widely according to the concentration of the agents, differences in individual sensitivity, and probably other factors. Expressed as percentages of control averages, the cells adjoining the ventral yolk border (A-cells) range from 12.9 in the highest azide concentration to 81.3 in the lowest cyanide concentration. Individual averages for all visible migratory cells (AB-cells in the tables) were determined only for the lower azide concentrations used. Expressed as percentages of control averages, they range from 47 to 64.8. For the different cyanide concentrations used, they range from 34.3 to 81.3 per cent of control averages.

3. Cells which become visible only partway down to the yolk border (B-cells) are relatively few in number but may be more numerous under the inhibiting conditions than in controls, particularly in cyanide, where they range up to 258.1 per cent of control averages.

4. Various lines of evidence discussed in the text indicate that the decreases in numbers of visible pigment cells in the region where counts were made are due, at least in part, to inhibition of migration rather than merely to delay or prevention of pigment formation in some cells which consequently remain invisible.

5. The origin of the melanoblasts from the extreme high end of the dorsiventral gradient, the probable high activity of the migratory cells, and the almost complete absence of any other effects of the agents on development, except retardation, all favor the conclusion that these cells are highly susceptible to the inhibiting conditions and that this susceptibility is definitely related to the gradient pattern of the embryo and larva.

6. Separate individual counts of right and left sides serve as examples of variations in numbers of cells on the two sides of the individual and in different individuals. Recounts in the agents and after 8 days in water for recovery show no distinct increase in cell numbers and indicate that first counts and recounts were fairly accurate.

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# GONADOTROPIC ACTIVITY OF BLOOD SERUM FROM HYPOPHYSECTOMIZED FROGS, RANA PIPIENS[1]

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Among the several functions of the anterior pituitary gland, the secretion of hormones which maintain the gonads is of paramount importance. Normal reproductive behavior is ordinarily associated with a healthy pituitary, and hypophysectomy is followed generally by a decline in activity of the ovary or testis. It is somewhat surprising, then, to find that hypophysectomy in the immature rat (Lane and Greep, 1935) and adult frog (Rugh, 1939) is followed by a pronounced, though temporary, increase in responsiveness of the ovary, evidenced in the rat by increase in weight of the ovary and in the frog by a greater percentage of ovulation after injection of pituitary material than that observed in normal frogs given an equivalent injection.

Lane and Greep attribute their results directly to the mechanics of hypophysectomy, suggesting that an extra surge of gonadotropins is sent into the blood stream as the gland is approached and removed. In Rugh's experiments an average of 52 per cent ovulation was obtained in four hypophysectomized *Rana pipiens* following the injection of one anterior pituitary gland. This same dosage produced no ovulation whatsoever in normal females or in animals subjected to sham hypophysectomy, an operation in which an opening is made in the basisphenoid bone, meninges pierced, but the anterior lobe left in place. These findings led Rugh to suggest that the frog's intact pituitary gland may exert an inhibitory influence on ovulation and that hypophysectomy removes this inhibition.

The senior author (Wright, 1946) made a more thorough study of the relation of hypophysectomy to ovulation in the frog, confirming and extending Rugh's observation that the ovary of the hypophysectomized animal is more readily stimulated. Contrary to Rugh's results, however, sham-operated animals with functional anterior lobes behaved exactly as completely hypophysectomized frogs in their ovulatory responses. Evidence for an inhibitor was therefore lacking in Wright's experiments, and he explained resulting data, as had Lane and Greep, on the basis of a gonadotropin released during hypophysectomy. In view of the work of Wright and Hisaw (1946), who showed a pronounced sensitizing effect of the follicle-stimulating hormone (FSH) on the frog ovary, the suggestion was made that FSH was set free during hypophysectomy, conveyed via the blood stream, and thus accounted for the increased responsiveness. Experiments were designed to test this hypothesis more fully, i.e., to attempt to demonstrate that blood of hypophysectomized frogs contains a gonadotropin similar in action to FSH. A description of these experiments, together with results, follows.

## MATERIAL AND METHODS

Large (50–100 gm.), sexually mature female frogs (*R. pipiens*), obtained di-

[1] Assisted by a grant from the Horace H. Rackham School of Graduate Studies, University of Michigan.

rectly from hibernation, were used in all experiments. After arrival in the laboratory the animals were stored at 1°–4° C. in pans containing a small amount of water. The smaller frogs (50–70 gm.) were satisfactory as subjects for hypophysectomy and as sources of blood, but ovaries for *in vitro* work were obtained from only the larger animals (65–100 gm.). For the sake of uniformity, only left ovaries were used, and one ovary supplied ample material for all tests assembled on any given day.

Hypophysectomy was performed through the roof of the mouth under light ether anesthesia by drilling a hole in the basisphenoid bone with a 2-mm. trephine, puncturing the meninges, and aspirating the anterior pituitary lobe into a pipette. Bleeding was ordinarily very slight, and the animals recovered rapidly from the effects of the anesthetic. No attempt was made to suture the oral mucosa after the operation, since a knot of thread seemed to irritate the animal unduly. Frogs were kept at room temperature following hypophysectomy.

After posteriorly pithing the frog, blood was obtained directly from the conus arteriosus through a hypodermic needle (No. 24) inserted at the primary branching point of the aorta. With the aid of a syringe, 1½–2 cc. of whole blood were obtained from each frog, and the animal was then discarded. Serum was separated by centrifugation at 4° C. and was also stored at that temperature.

Since individual pituitary glands vary somewhat in potency, concentrated stock solutions of fresh anterior pituitary glands from adult female frogs were used throughout the work. Stock solutions consisted of 15–20 glands finely macerated and taken up in a small volume of Ringer's solution. Dilutions for use *in vitro* were made readily from these concentrated preparations, which were kept frozen to prevent deterioration while not in use.

The technique for *in vitro* experiments was essentially that described previously (Wright, 1945). In brief, excised ovaries were cut into small fragments with 15–30 eggs, and these fragments were suspended by cotton thread in stoppered vials containing blood serum or pituitary extract diluted in Ringer's solution to a total volume of 10 cc. per vial. In every series of tests, six vials of each experimental or control medium were assembled. Thus the percentage of ovulation recorded for each test represents an average of values obtained in six preparations. Tabulations of percentages were made readily, since ovulated eggs (free of the ovary and resting on the bottoms of the vials), as well as those remaining in the ovarian stroma, could be counted accurately at any time during the course of the experiment.

## EXPERIMENTAL PROCEDURE AND RESULTS

Preliminary tests showed that blood serum from normal or hypophysectomized frogs, either full strength or diluted in Ringer's solution, was incapable of causing ovulation in ovarian fragments *in vitro*. To detect any possible gonadotropic activity of serum from hypophysectomized frogs, it was necessary, therefore, to use serum in conjunction with pituitary extracts and to note any modifying effect that the serum had on the amount of ovulation achieved in pituitary solution. It did not seem feasible to add serum directly to the pituitary-Ringer ovulating medium, however, since the presence of practically any protein material in the ovulating medium increases the percentage of ovulation beyond that obtained with pituitary alone (Wright, 1945). The use of a sequence of the two media was indicated, and a procedure which had been used previously

to demonstrate the sensitizing activity of FSH (Wright and Hisaw, 1946) was found satisfactory. This procedure consisted of keeping ovarian fragments in Ringer's solution with the desired amount of blood serum for 6–7 hours and transferring them after this time to pituitary solution, where ovulation ensued.

In order to have each experiment properly controlled, four types of preparations were assembled: (1) *Pituitary controls*. It was essential to know for each experiment the natural ovulatory capacity of ovarian tissue as it was removed from the frog. These preparations consisted of bits of ovary kept from the time that the ovary was excised in vials, each containing the equivalent of one-sixteenth of an anterior pituitary gland in 10 cc. Ringer's solution. In 24–28 hours the natural response of each ovary to that dosage of pituitary material *in vitro* was demonstrated. (2) *Ringer controls*. Since Ringer's solution was used as the medium to which blood serum was added, it seemed important to determine the effect of several hours' immersion in Ringer's solution alone. Fragments of ovary were placed in vials containing 10 cc. Ringer's fluid and were removed to pituitary solution at the same time as other fragments which had been immersed in Ringer containing blood serum. (3) *Normal serum controls*. To evaluate the gonadotropic activity of serum from hypophysectomized females, it was necessary to determine the effect of blood serum obtained from normal frogs. In doing this, fragments of normal ovary were suspended in vials, each containing 10 cc. Ringer's fluid plus 0.02 cc. normal serum and were transferred to pituitary solution after 6–7 hours, where ovulation was observed. (4) *Experimental serum preparations*. These were identical in all respects to normal serum controls just described, except that serum (again 0.02 cc. per vial) from hypophysectomized females was used in place of serum from normal animals. Serum for these tests was withdrawn at the following times after hypophysectomy: (*a*) within 10 minutes, (*b*) 2 hours, (*c*) 18 hours, (*d*) 96 hours, and (*e*) 9 days. Serum was also obtained from two animals 2 hours after they had been subjected to sham hypophysectomy. A fifth type of preparation, using FSH, was assembled on several occasions for comparison with serum of hypophysectomized animals. These consisted of ovarian fragments kept in vials containg 1 mg. of FSH[2] dissolved in 10 cc. Ringer's fluid and transferred to pituitary solution at the same time as other fragments which had been exposed to blood serum.

It seemed important to include these four types of preparations in each experiment because of the following considerations. Since the equivalent of one-sixteenth of an anterior pituitary gland in 10 cc. Ringer's solution was to be used as the ovulating medium throughout the work, it was essential to know the natural capacity of each ovary to respond to this dosage. Pituitary controls served this purpose, since no treatment was given these ovarian fragments other than placing them in pituitary solution as soon as possible after the ovary was excised. In Ringer controls ovarian tissue was deprived of contact with either pituitary factors or constituents of the blood for 6–7 hours before being placed in pituitary solution. Judging from previous experience (unpublished), it was expected that percentages of ovulation in Ringer controls (achieved after fragments were placed in pituitary solution) would fall short of those obtained in pituitary controls. The explanation of this decreased responsiveness is not clear, but it is

[2] FSH was supplied by Schering Corporation and was assayed at 20 synergist units per milligram.

thought that a certain amount of leaching of nutrient materials occurs during several hours of "soaking" in Ringer's fluid, and reduction in nutrient supply may contribute to the decreased responsiveness observed. As mentiond above, normal serum controls served to demonstrate any possible gonadotropic activity of normal blood serum. These preparations were the essential controls in each experiment, and a comparison of percentages of ovulation obtained in normal serum controls with those achieved in ovarian fragments exposed to serum from hypophysectomized females was the criterion for determining whether hypophysectomized frog's serum had increased gonadotropic activity. If several hours' contact with serum from hypophysectomized frogs effected consistently greater ovulatory responses after fragments had been transferred to pituitary solution than responses which followed exposure to normal serum, it would be logical and fair to ascribe increased gonad-stimulating action to blood serum of hypophysectomized females, but not otherwise.

After fragments of ovary had remained in Ringer-serum mixtures for 6–7 hours, the tissue was removed to fresh Ringer's solution, rinsed thoroughly, and placed in vials, each containing the equivalent of one-sixteenth of an anterior pituitary gland in 10 cc. Ringer's solution. Pieces of ovary in Ringer controls, as well as those which had been kept in FSH solution, were likewise washed simultaneously and placed in identical pituitary solutions. Vials were kept tightly stoppered except during transfer of tissue to prevent evaporation and possible excessive bacterial contamination. No ovulation occurred while ovarian fragments remained in any vials containing serum, FSH, or Ringer's solution and did not commence until 8–10 hours after change to pituitary solution. Final readings of percentages of ovulation were obtained 24 hours after the tissue had been placed in pituitary solution, since it was found that activity generally ceased at about that time.

All data are reported in Table 1, and a set of averages has been constructed in Table 2. It will be noted that ovarian fragments after 6–7 hours in Ringer's solution were noticeably less susceptible to pituitary factors (av. ovulation, 34.9 per cent) than were pieces of the same ovary put into pituitary solution at the beginning of the experiment (av. ovulation, 62.9 per cent). Percentages of ovulation after exposure to normal serum in Ringer's fluid, followed by pituitary solution, were not significantly greater (av. ovulation, 36.5 per cent) than those obtained in Ringer controls. However, percentages of ovulation following exposure to serum taken 2 and 18 hours after hypophysectomy, 2 hours after sham hypophysectomy, and to FSH were significantly higher than values obtained in respective normal serum controls. Blood serum withdrawn within 10 minutes after hypophysectomy, as well as that taken 96 hours and 9 days after the operation, were less effective or completely ineffective, and a possible explanation for their failure will be presented below.

Several tests were performed using differing amounts of serum (ranging from 0.005 to 0.5 cc. per 10 cc. Ringer) and allowing fragments of ovary to remain in Ringer-serum mixtures for shorter and longer periods of time (from 4 to 16 hours). Results showed, however, that 0.02 cc. serum (withdrawn 2 hours after hypophysectomy) per vial and 6–7 hours' exposure to serum gave the most satisfactory values. Dosages of serum less than 0.02 cc. per vial failed to maintain the ability of the ovary to respond

## TABLE 1*

RESULTS OF *in vitro* EXPERIMENTS SHOWING PERCENTAGES OF OVULATION ACHIEVED IN VIALS EACH CONTAINING A FRAGMENT OF FROG OVARY AND THE EQUIVALENT OF ONE-SIXTEENTH FROG ANTERIOR PITUITARY GLAND IN 10 CC. RINGER'S SOLUTION

| DATE | OVULATION IN | | | HOURS IN RINGER, FSH, OR BLOOD SERUM | OVULATION AFTER EXPOSURE TO BLOOD SERUM TAKEN THE FOLLOWING TIMES AFTER HYPOPHYSECTOMY | | | | | | FSH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pituitary Controls | Ringer Controls | Normal Serum Controls | | 0 Hour | 2 Hours | 2 Hours after Sham Hypophysectomy | 18 Hours | 96 Hours | 9 Days | |
| 1-12 | 86.9 | 1.7 | 5.2 | 6½ | ..... | 11.3 | ..... | ..... | ..... | ..... | ..... |
| 1-15 | 44.1 | 17.5 | 15.9 | 6 | ..... | 25.0 | ..... | ..... | ..... | ..... | ..... |
| 1-17 | 47.3 | 54.7 | 55.6 | 7 | ..... | 80.0 | ..... | ..... | ..... | ..... | ..... |
| 1-18 | 46.2 | 53.5 | 65.4 | 6 | ..... | 68.3 | ..... | ..... | ..... | ..... | ..... |
| 1-19 | 14.4 | 3.7 | 4.7 | 6 | ..... | 10.8 | ..... | ..... | ..... | ..... | ..... |
| 1-20 | 42.7 | 25.9 | 43.3 | 6 | ..... | 54.6 | ..... | ..... | ..... | ..... | ..... |
| 1-26 | 95.1 | 12.7 | 19.8 | 7 | ..... | 54.7 | ..... | ..... | ..... | ..... | 63.6 |
| 2-2 | 44.7 | 8.8 | 10.8 | 6 | ..... | 33.3 | ..... | ..... | ..... | 8.5 | 34.4 |
| 2-9 | 25.9 | 6.3 | 4.8 | 6 | ..... | 17.2 | ..... | ..... | ..... | 5.3 | 17.0 |
| 2-16 | 55.5 | 38.9 | 36.0 | 6 | ..... | 60.4 | 83.6 | ..... | ..... | 33.3 | 75.0 |
| 2-18 | 39.8 | 9.3 | 16.5 | 6 | ..... | 24.8 | 30.7 | ..... | ..... | ..... | ..... |
| 2-21 | 85.6 | 30.6 | 25.4 | 6 | ..... | 31.9 | 42.2 | ..... | ..... | ..... | ..... |
| 2-22 | 83.9 | 76.3 | 79.3 | 6 | ..... | 81.6 | 85.0 | ..... | ..... | ..... | ..... |
| 2-23 | 84.8 | 35.1 | 56.0 | 6 | 50.0 | ..... | ..... | ..... | ..... | ..... | ..... |
| 2-25 | 85.7 | 64.8 | 60.7 | 6 | 69.7 | ..... | ..... | ..... | ..... | ..... | ..... |
| 2-26 | 24.3 | 13.1 | 21.2 | 6 | 20.8 | ..... | ..... | ..... | ..... | ..... | ..... |
| 3-7 | 96.2 | 69.0 | 50.4 | 6 | ..... | ..... | ..... | 72.2 | ..... | ..... | ..... |
| 3-9 | 93.8 | 80.9 | 79.9 | 6 | ..... | ..... | ..... | 90.8 | ..... | ..... | ..... |
| 3-11 | 63.8 | 21.6 | 19.5 | 6½ | ..... | ..... | ..... | 7.6 | 22.4 | ..... | ..... |
| 3-14 | 68.7 | 47.4 | 38.7 | 6 | ..... | ..... | ..... | 62.6 | 52.0 | ..... | ..... |
| 3-16 | 92.7 | 62.6 | 58.4 | 6½ | ..... | ..... | ..... | 66.7 | 64.0 | ..... | ..... |

* Each value represents an average percentage obtained in six similar vials. Pituitary controls consisted of bits of ovary placed in pituitary solution at the beginning of the experiment and left undisturbed. Bits of ovary in Ringer controls were kept in Ringer's fluid for the number of hours shown and then transferred to pituitary solution. In normal serum controls and all experimental preparations, enumerated in columns to the right above, fragments of ovary were placed in vials, each containing 0.02 cc. blood serum in 10 cc. Ringer's solution and changed to pituitary solution after the period shown. One mg. FSH per vial was substituted for blood serum in obtaining the results shown in the last column.

## TABLE 2*

AVERAGES OF PERCENTAGES OF OVULATION SHOWN IN TABLE 1

| Blood Serum | Pituitary Controls | Ringer Controls | Normal Serum Controls | Experimentals | Δ Fifth Col.– Fourth Col. |
|---|---|---|---|---|---|
| 0-hour | 64.9 | 37.7 | 46.0 | 46.8 | + 0.8 |
| 2-hour | 54.8 | 26.2 | 29.4 | 42.6 | +13.2 |
| 2-hour(sham) | 66.2 | 38.8 | 39.3 | 60.4 | +21.1 |
| 18-hour | 83.0 | 56.3 | 49.4 | 60.0 | +10.6 |
| 96-hour | 75.1 | 43.9 | 38.9 | 46.1 | + 7.2 |
| 9-day | 42.0 | 18.0 | 17.2 | 15.7 | − 1.5 |
| FSH | 55.3 | 16.7 | 17.9 | 47.5 | +29.6 |

* The differences (Δ) between values obtained in experimental preparations (fifth col.) and those achieved in normal serum controls (fourth col.) are given in the last column.

to pituitary factors, and greater amounts did not increase the responsiveness consistently.

## DISCUSSION

From these data there seems to be no question that gonadotropic activity has been demonstrated in blood serum of hypophysectomized frogs. It should be mentioned simultaneously with this statement, however, that serum demonstrating marked gonadotropic activity must be obtained within certain specified times following hypophysectomy. The appearance of gonadotropin in blood serum after hypophysectomy, at least that detectable by our method, follows a logical pattern. First, it will be noticed (Tables 1 and 2) that serum obtained 2 hours after hypophysectomy effected markedly increased percentages of ovulation, while serum withdrawn within 10 minutes postoperatively was essentially indistinguishable in its action from normal serum. Since at hypophysectomy the anterior lobe is torn loose from its vascular attachments, it does not seem likely that hormone released during the operation will find its way to the general circulation immediately. Blood withdrawn within a few minutes after hypophysectomy, then, could hardly be expected to contain more gonadotropin than that of normal animals. It is again logical to assume that gonadotropic hormone set free during hypophysectomy is absorbed into systemic circulation along with lymph and other extra-cellular fluid, so that its concentration in the blood stream reaches a maximum within 1 or 2 hours. After the peak of gonadotropic content in the blood has been reached, there follows a decrease, as the hormone is utilized and metabolism progresses. Serum taken 18 hours after hypophysectomy still possesses gonad-stimulating activity (in four of the five tests conducted, Table 1), but averages of percentages of ovulation (Table 2) suggest a lower gonadotropic content than in 2-hour serum. By 96 hours after hypophysectomy, blood serum is still somewhat more effective than normal serum, but by 9 days all traces of gonadotropic activity are gone.

The results presented are exactly in line with data previously reported (Wright, 1946) on responses of the hypophysectomized female frog to the injection of the equivalent of one pituitary gland. It was shown that the animal did not reach maximal sensitivity until some hours after hypophysectomy (100 per cent ovulation achieved when injected 16 hours after the operation). Ovulation obtainable 4 days after hypophysectomy was approximately 70 per cent, and at 9 days less than 10 per cent. Normal frogs ovulated an average of only 31 per cent of their eggs when given the same dosage of pituitary material.

It is interesting to note that serum from animals subjected to sham operations gave consistently higher ovulation values than serum taken from animals 2 hours after the completed operation. It is very probable that the anterior lobe in sham-operated animals continues to release gonadotropin for some time because of relationships established by the operation. Since a direct communication between pituitary gland and oral cavity is brought about by trephining, the gland is undoubtedly subjected to alternate increases and decreases in pressure associated with the animal's respiratory activity. Without question, there is greater opportunity for higher gonadotropin levels in the blood of these animals than in females completely hypophysectomized.

Data obtained in using FSH as a sensitizing agent compare favorably with percentages achieved after exposure to 2-hour serum. The similar action of these two agents suggests that serum from re-

cently hypophysectomized frogs contains an extra quantity of FSH. To the authors' knowledge, no investigator has extracted FSH as such from amphibian pituitary glands; but, since the effects of anterior lobe administration can be exactly duplicated with FSH and combinations of FSH and luteinizing hormone (LH) (Wright and Hisaw, 1946), the presence of these gonadotropins in the frog hypophysis is assumed. It is conceivable that some amount of both FSH and LH are set free during hypophysectomy. However, since as little as one-eighth unit LH accompanying one-sixteenth unit FSH causes ovulation in ovarian fragments *in vitro*, the amount of LH released at hypophysectomy must be extremely small, for no ovulation occurred in ovarian fragments left for 28 hours or more in 2-hour serum. Furthermore, if LH were released at hypophysectomy, some amount of ovulation would be stimulated in the operated animal. It appears, then, that only FSH can be released, for no hypophysectomized frog has been observed to ovulate spontaneously.

## SUMMARY

Blood serum of recently hypophysectomized female frogs was shown to have a marked gonadotropic action on pieces of frog ovary *in vitro*, 0.02 cc. serum in 10 cc. Ringer's solution producing maximal stimulation in 6–7 hours, after which time the progress of ovulation was observed in pituitary solution. Controls in which plain Ringer's solution was used in place of Ringer-serum mixtures gave consistently lower ovulation values, and the addition to Ringer's solution of 0.02 cc. per vial of serum from normal animals failed to increase the responsiveness.

Serums having the greatest gonadotropic activity were those taken 2 and 18 hours after hypophysectomy or 2 hours after sham hypophysectomy. Serum taken 96 hours after the operation had decreased gonad-stimulating action, and serum withdrawn 9 days after hypophysectomy was ineffective. Possible explanations for these differences are discussed.

One mg. of FSH added to 10 cc. Ringer per vial was shown to have gonadotropic activity similar to that of blood serum withdrawn from frogs 2 hours after hypophysectomy. Because of this similarity, it is postulated that 2-hour, 18-hour, and, to a lesser extent, 96-hour serums contain an amount of FSH greater than that presumably present in serum of normal frogs.

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# A CONSIDERATION OF THE PROTHROMBIN TIMES OF SEVERAL AMPHIBIANS, WITH NOTES ON EFFECTS OF PARASITIZATION AND DISEASE

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## INTRODUCTION

ALTHOUGH the coagulation of blood has been widely studied in avian and mammalian subjects, relatively little attention has been directed toward the phenomenon as it appears in the lower vertebrates. The Amphibia, however, have not been entirely neglected. The various investigations of Tait and his collaborators (cf. Tait and Green, 1926), as well as those of a few earlier workers cited by them, have brought forth certain information concerning the coagulation of blood in the frog. All amphibian blood thus far described has been found to contain delicate structures called "spindle cells," which appear to be analogous to the platelets of mammalian blood. Upon rupture of these spindle cells, fibrous clots similar to those of coagulated mammalian blood appear. As a part of a study of shock in *Rana pipiens*, Moreau, Balistocky, and Heilbrunn (1948) measured the clotting times of control and injured specimens by the capillary method.

In recent years "prothrombin time" (Quick, 1937) has become an acceptable index of the coagulative potency of mammalian and avian blood. Brambel (1941) applied the quantitative technique of Quick in studying the coagulation of turtle blood. In 1940 Kato developed a technique for measuring prothrombin times by microchemical methods. Because only small amounts of blood can be obtained from individual specimens of most species of Amphibia, Kato's technique was adopted and used in the present study with the modifications described below.

## MATERIAL AND METHODS

### ANIMALS

Blood samples from the following amphibians were examined: *Gyrinophilus porphyriticus porphyriticus* (Green) (northeastern purple salamander), *Necturus maculosus maculosus* (Rafinesque) (mudpuppy), *Triturus viridescens viridescens* (Rafinesque) (spotted newt), *Rana catesbeiana* (Shaw) (bullfrog), *R. clamitans* (Latreille) (green frog), *R. pipiens* (Schreber) (leopard frog), *Bufo americanus* (Holbrook) (American toad), and *Scaphiopus holbrookii* (Harlan) (spadefoot toad). The specimens of *R. catesbeiana* were collected in Louisiana, those of *R. pipiens* in Wisconsin, and those of *S. holbrookii* in West Virginia. The remaining forms were collected locally in western Pennsylvania.

### REAGENTS

The following reagents were utilized in the application of Kato's (1940) microtechnique for measuring prothrombin times.

1. *Solution A.*—Two hundred ml. of powdered thromboplastin extract (com-

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mercial extract obtained from the Difco Company and thromboplastin extract prepared in the laboratory from rabbit brain [Kato, 1940] were found to be equally effective) were mixed with 5 ml. of a 0.11 molar solution of sodium chloride (Kato's technique calls for normal saline solution) and put into an oven at 45°–50° C. for 10 minutes. The mixture was then shaken thoroughly for a few minutes and let stand at room temperature until the powder settled. The supernatant liquid (solution A) was decanted and either used immediately or frozen and later thawed when needed.

TABLE 1

MEAN* PROTHROMBIN TIMES FOR EIGHT SPECIES OF AMPHIBIA

| Species Tested | No. of Specimens | No. of Tests | Mean Prothrombin Times (Seconds) | Standard Deviations |
|---|---|---|---|---|
| *G. porphyriticus* | 10 | 72 | 27.6 | 2.73 |
| *N. maculosus* | 10 | 31 | 55.8 | 5.73 |
| *T. viridescens* | 42 | 42 | 31.8 | 9.30 |
| *R. catesbeiana* | 8 | 77 | 83.6 | 1.41 |
| *R. clamitans* | 6 | 18 | 81.0 | 3.03 |
| *R. pipiens* | 12 | 53 | 81.3 | 2.23 |
| *B. americanus* | 22 | 116 | 44.7 | 1.96 |
| *S. holbrookii* | 2 | 16 | 46.8 | 8.72 |

* Actually the mean of the means of the results obtained for individual specimens.

2. *Solution B.*—It was discovered that substitution of a fluoride solution (6.5 mg. calcium chloride and 12.0 mg. sodium fluoride per milliliter of water) for the double oxalate solution recommended by Kato is desirable because the oxalate solution frequently failed to prevent spontaneous coagulation of urodele blood samples.

3. *Solution C.*—Aqueous solution of calcium chloride (2.8 mg. calcium chloride per milliliter).

TECHNIQUE

Before blood samples were collected, each animal was anesthetized—the anurans with ether, the salamanders with chloretone (4 per cent aqueous solution). In each specimen the heart was exposed, and blood was extracted from the apex of the ventricle by means of a small glass pipette, the tip of which was drawn to an inside diameter about 200 $\mu$ and calibrated in 0.01-ml. units. Just before insertion into the ventricle, some of solution B was drawn into and expelled from the pipette so that enough of the solution remained adsorbed in the pipette to prevent spontaneous clotting when blood was taken into it.

One-hundredth ml. each of solutions A and C was measured with a serological pipette and put into the concavity of a depression slide. The two solutions were mixed thoroughly in the concavity with a small glass stirring rod. One-hundredth ml. of blood was then added to the above mixture, and stirring was continued for 5–10 seconds. A stop watch was used to measure the lapse of time between the addition of the blood to the mixed solutions and the appearance of a firm gelatinous clot. This period of time, beginning with the mixing of blood and reagents and terminating with clot formation, was called the "prothrombin time" by Kato and the term "prothrombin time" has the same meaning here.

OBSERVATIONS

Table 1 records the results of tests made on representatives of the eight species of Amphibia studies. In addition to the twelve apparently healthy specimens of *R. pipiens* cited in Table 1, parasitized animals and animals with "red leg" (Emerson and Norris, 1905; Rose, 1946), a disease common among laboratory frogs, were also examined and tested. When the described testing procedure

was applied to some seventy frogs infected with "red leg," truly definitive clots were obtained in none of the tests; however, small masses of solid material, which may have represented the clumping of cells in beginning clot formation, were occasionally seen in the tested samples. These masses never joined together to form the solid clots characteristic of tests made on blood from uninfected frogs. Blood extracted from frogs with "red leg" was also pale in color and appeared to be more watery than blood taken from typical individuals.

fected animals, stirred for 5–10 seconds, and the time required for clot formation noted. The results obtained are recorded in Table 2. The blood samples showed progressive decrease both in richness of color and in apparent viscosity during the course of the experiment until, finally, the samples of the ninth and tenth days were quite pale and watery and did not clot during the 4 minutes they were observed.

It was noted that some of the specimens of *R. pipiens* had unusually long prothrombin times but yet showed no

TABLE 2

MEAN CLOTTING TIMES OF 0.01-ML. SAMPLES OF BLOOD TAKEN FROM TEN SPECIMENS OF *R. pipiens* INFECTED TO APPROXIMATELY THE SAME DEGREE WITH "RED LEG" AND TESTED ONE PER DAY FOR 10 DAYS

| | Day Tested | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Mean clotting time in seconds | 62 | 74 | 71 | 90 | 98 | 150 | 153 | 153 | No clot in 240 sec. | No clot in 240 sec. |

Having made the observations just described and having noted that, when fatalities ensued among the diseased frogs, the onset of death was often accompanied by sustained hemorrhage, a brief experiment was conducted in an effort to find out whether blood from animals in advanced stages of the disease clotted more readily than did blood from animals showing only the early symptoms. Ten frogs, all in that early stage of the disease which is apparently characterized only by a slight redness of the underside of the hind limbs, were selected and tested; one each day for 10 days. Since blood from such frogs had previously failed to clot when diluted with reagents, 0.01-ml. samples of undiluted whole blood were removed from the in-

symptoms of "red leg." Upon dissection of these animals, it was discovered that they were parasitized by *Pneumoneces* (sp?), a species of lung fluke. Blood from nine of them was tested, and the number of flukes found in each was recorded (Table 3). It will be seen that in each of these animals the prothrombin time was well above the mean prothrombin time cited in Table 1 for typical individuals of this species. Table 3 also gives the results of tests made on three specimens of *B. americanus* which were parasitized with lung flukes (unidentified species). As in *R. pipiens* the prothrombin times of the parasitized animals were considerably greater than the mean prothrombin time of the uninfested individuals (cited in Table 1).

## DISCUSSION

The results obtained for *T. viridescens* indicate a wide range of individual variations in clotting time; but, as may be deduced from the relatively low standard deviations cited for the other mean prothrombin times (except that for *S. holbrookii*, for which the results cover only two specimens), the observed prothrombin times are fairly constant within each species.

Making allowances for the variations observed in *T. viridescens* and excepting the data on *N. maculosus*, it is apparent that the species studied fall into three groups with regard to prothrombin time: one group includes the urodeles; another the frogs; and the third the toads. Application of a formula for comparison of the means of small samples (Simpson and Roe, 1939) shows the three groups of means to be significantly different. The number of species represented here is too small to support a final conclusion concerning the phylogenetic significance of prothrombin time. The results do, however, suggest that prothrombin times, like many other biochemical characteristics (Florkin, 1949), are peculiar for the various animal groups.

The observations made on frogs infected with "red leg" clearly show that the efficiency of the clotting mechanism of frog blood is reduced by the disease. It should prove interesting to test the effects of administering vitamin K to frogs carrying "red-leg" infections.

The secretion of anticoagulins by helminth parasites has apparently been only briefly considered by physiologists. McCoy (1935) cites only three papers on the subject, and all three deal with nematodes. That the flatworms infesting the anurans studied here release an anticoagulating factor or factors is strongly indicated by the results given in Table 3.

TABLE 3

MEAN PROTHROMBIN TIMES OF ANIMALS INFESTED WITH LUNG FLUKES

| Animal No. | *R. pipiens* | | Animal No. | *R. pipiens* | | Animal No. | *B. americanus* | |
|---|---|---|---|---|---|---|---|---|
| | No. of Flukes | Mean Prothrombin Time (Seconds) | | No. of Flukes | Mean Prothrombin Time (Seconds) | | Mean Prothrombin Time (Seconds) | |
| 1 | 6 | 99.0 | 6 | 12 | 101.0 | 1 | 54.4 | |
| 2 | 8 | 99.5 | 7 | 13 | 104.0 | 2 | 83.1 | |
| 3 | 9 | 91.0 | 8 | 17 | 166.0 | 3 | 63.0 | |
| 4 | 9 | 146.0 | 9 | 23 | 101.0 | | | |
| 5 | 10 | 92.0 | | | | | | |

## SUMMARY

1. The prothrombin times of eight species of Amphibia were measured: *G. porphyriticus* (27.6 sec.), *N. maculosus* (55.8 sec.), *T. viridescens* (31.8 sec.), *R. catesbeiana* (86.3 sec.), *R. clamitans* (81.0 sec.), *R. pipiens* (81.3 sec.), *B. americanus* (44.7 sec.), and *S. holbrookii* (46.8 sec.).

2. Wide individual variation in prothrombin time was observed among the specimens of *T. viridescens*, but fairly constant values were obtained for the other species, excepting *S. holbrookii*, in which only two specimens were examined.

3. A possible phylogenetic significance of prothrombin time is suggested.

4. Blood from frogs infected with "red leg" failed to clot when the testing technique standardized for this investigation was applied.

5. Frogs parasitized with lung flukes had elevated prothrombin times.

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# THE SELECTED TEMPERATURE OF ATLANTIC SALMON AND SPECKLED TROUT AND THE EFFECT OF TEMPERATURE ON THE RESPONSE TO AN ELECTRICAL STIMULUS[1]

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MORE than fifty years ago (Mendelssohn, 1895) it was first clearly recognized that, when motile organisms are placed in an environment in which there is a gradient of temperature, they tend to congregate in a restricted range of temperature which is well within the lethal limits. During the intervening years it has become very well established that organisms of all kinds, from Protozoa to mammals, exhibit this behavior (see, e.g., Jennings, 1904; Loeb, 1918; Herter, 1940; Fraenkel and Gunn, 1940). Several different names have been proposed for it. The term "temperature selection" will be used throughout the present work. The organism, or organisms if several are involved, will be said to "select" the temperature in question, and the latter will then be known as the "selected" temperature.

Although the fact of temperature selection has been known for a relatively long time, very little investigation has been made of the physiological processes by which organisms move to the selected region and then remain there. Locomotion is, of course, involved in the behavior, and it is evident that temperature must affect the individual movements of which locomotion is made up, in such a

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way that they result in a *net* movement toward some particular region, which is then said to be "selected."

Now the translocatory movements of an organism, in so far as they determine the position of the organism in space or in a temperature gradient, may be completely described in terms of their direction, length, and duration in time. There are, in fact, no other fundamental terms for such a description. When congregations of organisms occur in some particular region, therefore, it follows that one or more of these three attributes of the movements have been affected in such a way that the net effect of locomotion is movement toward the region in question. Temperature selection, then, must result from some effect of temperature on the direction, length, or duration of individual movements.

With the above very general considerations in mind, it is interesting to find that temperature affects the length of the movement made by trout in response to an electrical stimulus (Elson, 1942). When confined to a long narrow trough, this organism responds to the discharge of a condenser through the water by making a reasonably discrete "dart," whose length can be measured readily. Temperature affects this response, and in the trout used by Elson the average length of the dart was a maximum at approximately 10° C. Recognizing that some such effect of temperature of the individual translocatory movements of the organism must be the immediate cause of temperature selection, one would suspect a correspondence of some sort between the temperature selected and the effect of temperature on the individual movements. In the case of trout it was considered possible that the temperature 10° C. at which the darts were longest might also be the temperature selected by the organisms in a gradient of temperature. We have therefore determined the latter, the observations made being discussed below.

In addition, both the selected temperature and the effect of temperature upon the distante moved in response to a stimulus have been determined for a second species, the Atlantic salmon.

## MATERIALS AND METHODS

The experiments on the distance moved by salmon and trout when stimulated were carried out in a manner identical with that described previously for trout (Elson, 1942). In the present case, however, the longitudinal trough employed had glass sides, and the organisms were observed from the side rather than from the top. The individual salmon parr and the trout fingerlings which were used were approximately one year old and 10 cm. in length. For at least 1 month prior to these experiments they had been kept in tanks in the laboratory at a temperature of approximately 4° C.

The organisms used for the experiments on temperature selection were fry in which the yolk sac had been practically completely adsorbed and which were beginning to feed. These had been developed in the laboratory from eggs at approximately 4° C.

Salmon (*Salmo salar*, Linnaeus) were obtained from the Dominion Department of Fisheries by courtesy of Mr. Rodd at Ottawa and Mr. Catt at St. John. The trout (*Salvelinus fontinalis*, Mitchill) came from the private hatchery operated by Mr. Herbert Pearen at Horning's Mills, Ontario. We acknowledge the co-operation of these individuals with thanks.

The temperature gradient employed for the selection experiments was established in a trough cut in a cylindrical copper rod, 2 inches in diameter, which was electrically heated at one end and was

cooled at the other by mixtures of ice and salt. A channel 56 inches long, 1 inch deep, and $\frac{3}{4}$ inch wide was cut into the rod, thus forming a longitudinal chamber in which the organisms were placed. The surfaces of the metal in the channel were painted white, and for these experiments the channel contained water to a depth not exceeding $\frac{1}{2}$ inch. Temperature was measured by small copper-constantan thermocouples, which projected through the glass cover of the channel to within $\frac{1}{4}$ inch of the bottom. These thermocouples were spaced 4 inches apart, and the position of the fish at any moment desired was recorded with reference to them. The copper rod was well heat-lagged so that the temperature gradient would be as even as possible. In general the temperature varied from 0° C. at the cold end to 30° C. at the warm end.

We wish to record, with thanks, that this equipment for experiments on temperature selection was made available to us by courtesy of Dr. Norma Ford Walker, for whom it had been built several years previously.

## RESULTS

### 1. EFFECT OF TEMPERATURE ON THE DISTANCE MOVED BY SALMON PARR IN RESPONSE TO STIMULUS

When a condenser is discharged through a long narrow tank of water containing a salmon parr, this organism responds to the electric current, just as trout fingerlings have been reported to do (Elson, 1942), by moving forward.

It is easily observed visually that this movement consists of a vigorous primary "dart," followed by one or more much less vigorous secondary "darts." Each of the latter commences just before movement from the preceding one is quite ended. Typically, the amount of movement forward during the secondary darts is only a small fraction, not over 25 per cent, of that occasioned by the primary dart. It is relatively easy, as a consequence, to determine the position of the fish corresponding to the end of the distance traveled in the primary dart and hence to measure that distance itself. In what is to follow, in fact, attention will be confined to the distance traveled in the primary dart. Occasionally, a fish will change its direction of orientation immediately after the application of a stimulus and then will proceed to move forward. These responses were not included in the considerations of this report. Similarly, because it was not usual, we will omit from consideration at this time a type of response in which a degree of "refractoriness" was exhibited by the organisms. This occurred occasionally in almost all organisms, and in some was quite typical. The dart appeared to commence in response to the stimulus, but, before more than a small fraction of an inch had been traveled, it was brought suddenly to a halt, so that the animal rocked forward on the axis between its pectoral fins. The resultant motion was strikingly similar to that which occurs in a motorcar when the brakes are applied suddenly. Such responses were simply omitted when the average distances per dart were computed.

The distance moved by salmon in response to the electrical stimulation was found to vary, as in trout, with the strength of the stimulus, i.e., with the voltage to which the condenser was charged, and also with the temperature. Typical data showing the distance moved as a function of the stimulus strength at several temperatures are shown in Figure 1. The fact that the stimulus strength had to be above a fairly definite threshold and that the response tended to approach a maximum is clearly shown.

In a general way these curves undoubtedly indicate that the actual excitable elements stimulated here were not all equally excitable. As the stimulus strength was increased, more and more of them were excited until finally all were involved, and hence a maximum response was observed. It is reasonable to expect that the response curves in Figure 1 represent the integral frequency distribution of these excitabilities. If the excitabilities of these elements were distributed normally with respect to voltage, then each of the sets of observations in Figure 1 should conform to an ogive, which is the integral form of a normal distribution. The curves indicated by the solid lines drawn through the observations in the figure are such theoretical ogives. It is readily apparent that they represent the data reasonably well, although there is possibly a slight tendency for the responses at low stimulus strengths to be greater than the normal distribution requires.

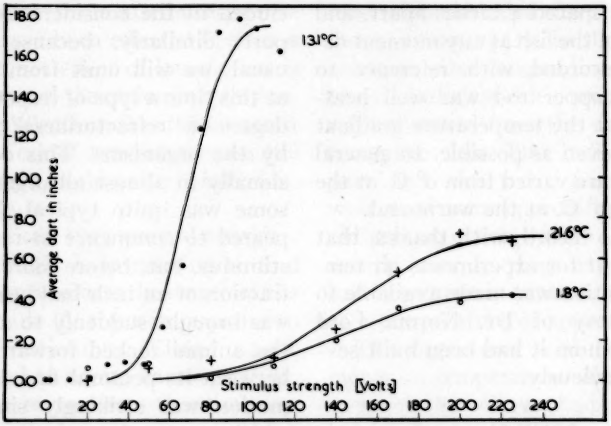


FIG. 1.—Typical data (single experiments) illustrating at three different temperatures the relation between the strength of the stimulus and the size of the response.

It is appropriate at this point to note that the electrical resistance of the water in the trough decreases, of course, as the temperature rises, thus decreasing the time constant of the condenser discharge circuit. The duration of the stimulus will consequently become less as the temperature rises. In general, unless the duration is so great that the stimulus strength is essentially at the rheobasic level, the effectiveness of a stimulus varies with its duration. It could be expected, therefore, that the effectiveness of a given stimulus in the present experiments might become less as the temperature rises. Theoretically at least, therefore, some part of the effect of changing the temperature could have resulted from the purely physical relation between temperature and the electrical resistance of the water.

In order to obtain an estimate of the magnitude of the effect which the duration of the stimulus might have, a few experiments were made in which the responses to stimuli of two different durations were compared at constant temperature. These experiments were made in the usual way, but the stimulating circuit was so arranged that either a 1- or a 0.3-microfarad condenser could be em-

ployed. It was the practice to determine the response to the stimulus from one condenser, then to raise the voltage to the next value to be tested, and to determine the response with the second condenser. The first condenser was employed again for the next higher voltage, the second for the fourth, and so on. The total number of observations made was therefore exactly the same as in the usual single series, but half were obtained with one condenser and half with the other. Two typical sets of data will be found in Figure 2. It is very apparent in both cases that, for a given response, a higher voltage was required, in general, when the smaller condenser was used than when the larger condenser was employed. The magnitude of the *maximum* response, however, was probably the same for both condensers. The data are not numerous enough to establish this latter point, but, on the basis of existing knowledge about excitation in isolated tissue, it is the finding to be expected.

The difference between the time constants employed in Figure 2 was, of course, much greater than would be produced by the change of temperature involved in the present experiments. The resistance of water increases by only 50 per cent, in fact, as the temperature is lowered through the range studied. The ratio of the time constants at the two extremes of temperature is therefore only $1\frac{1}{2}$, as compared with the ratio of the two constants in Figure 2, which is $3\frac{1}{3}$. Nonetheless, it is evident that for the most precise work care must be taken to insure that changes in the electrical resistance of the water do not distort the result.[3]

[3] It should perhaps be noted here that these considerations do not in any way invalidate the original conclusions of Elson (1942), first, because preliminary experiments had suggested that the large condenser (4.0 microfarads) used resulted in stimuli of nearly rheobasic strength: and, second, because the practically linear change of the time constant with temperature could not of itself produce the maximum response seen at intermediate temperatures.

The artifact which changes in the resistance might introduce can be greatly minimized, if not entirely removed, in experiments of this kind by (1) the use of a condenser large enough to insure that all stimuli are of rheobasic strength; (2) comparing only maximum responses; or (3) adjusting the time constant electrically. In the present instance any difficulty will be avoided by confining attention solely to the maximum response of the organisms at each temperature. The latter was determined from data similar to that shown in Figure 1. Exclusive of a number of preliminary trials, three identical experiments were made with trout and a similar three identical experiments with salmon parr. In each case observations were made at approximately 2, 5, 10, 13, 17, and 21° C., a different random sequence of these temperatures being employed in each experiment. The observations at any one temperature required 2–3 hours, and each complete experi-

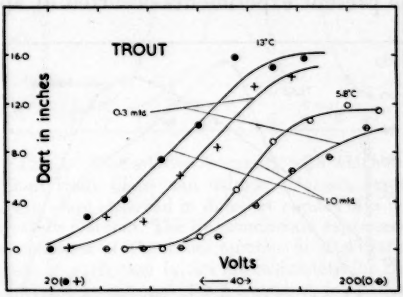


FIG. 2.—Typical data (single experiments) illustrating the relation between the strength of the stimulus and the size of the response. At each of the two temperatures employed, observations were made, using both a 1- (*dots and circles*) and a 0.3-microfarad condenser (*crosses and "barred" circles*). To avoid confusion, the data obtained at the lower temperature were moved to the right on the horizontal axis with respect to the data for the higher temperature.

ment involving all six temperatures extended over 2–3 days. The average response of trout at each temperature will be found in Figure 3, along with equivalent averages from the experiments with salmon. The original observation that the distance moved by a trout in response to an electrical stimulus is a maximum at a temperature near the middle of the range tolerated by the organism was completely confirmed. The maximum in the present experiments occurred at approximately 10° C., the organisms having been acclimated to approximately 4° C.

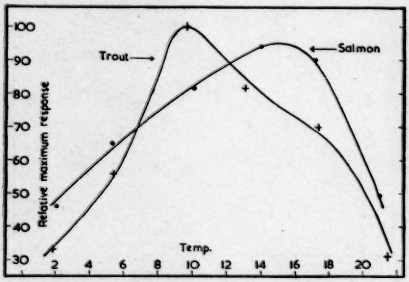


FIG. 3.—The maximum distance moved by salmon (*dots*) and trout (*crosses*) in response to electrical stimuli at different temperatures. For simplicity in each complete experiment involving the six temperatures, the largest average response was taken as 100, the responses at the other temperatures being expressed as percentages of this. The three experiments for each form were then respectively averaged so as to obtain the data given in this figure.

The figure shows, in addition, that the responses of salmon, too, passed through a maximum. This occurred at approximately 15° C. and therefore at an appreciably higher temperature than that at which the response of trout was a maximum. It was noted that the difference between trout and salmon existed even at stimulus strengths lower than those which evoked a maximum response, although, for the reasons set forth above, comparisons at stimulus strengths below those which produce a maximum response may not be quite so significant in the present instance as are those based on a maximum response.

### 2. TEMPERATURE SELECTION

The procedure for the experiments in the temperature gradient was essentially as follows: first, the gradient was established; 13–17 organisms of either species were then introduced, and, after approximately an hour, observations were commenced. At intervals of approximately 15 minutes the temperature of each thermocouple was determined, and the position of each organism with respect to the various thermocouples was recorded. Usually the organisms were intentionally disturbed immediately following an observation either by causing a shadow to pass over them or by touching them with a glass rod. This was done so that a new "selection" would be made after each observation. From the data thus accumulated the frequency with which organisms were found at different temperatures was obtained.

The duration of the experiments varied from 7 to 25 hours. In every experiment the distribution during the first half of the experiment was compared with that during the last half. There was no evidence of a consistent difference between the two halves, and in several instances there was practically no difference at all between them. Furthermore, the experiments with trout and those with salmon did not appear to differ among themselves. The data of the three identical experiments for each species were therefore combined, and the resulting frequency curve will be found in Figure 4. It is very apparent that both forms selected temperature and that the temperature selected is approximately 10° C. in the case of trout but 14° C. in the case of salmon.

A quantitative statistical test of the

significance of the difference observed in the distributions of the trout and salmon in the temperature gradient is of some interest. Several tests exist, some of which require the presumption that the distributions under consideration are "normal" in the statistical sense, while others do not. Application of a conventional test for the normality of a distribution (Chambers, 1945, p. 27) indicated that the distributions shown in Figure 4 do not differ significantly from normal curves, so that actually any of the tests for the significance of differences could have been employed. As a generally applicable procedure, however, it seemed better to use a test which is independent of the exact nature of the distributions to be compared. Such a one is that involving the statistic $\chi^2$ (Chambers, 1945). To apply it in the present instance the value of $\chi^2$ was calculated, proceeding from the hypothesis that the two sets of data, salmon and trout, were samples of each individual experiment. The probability that the value of $\chi^2$ found would occur by chance if the sets of data compared were samples of the same population of data will be found in column 4 of

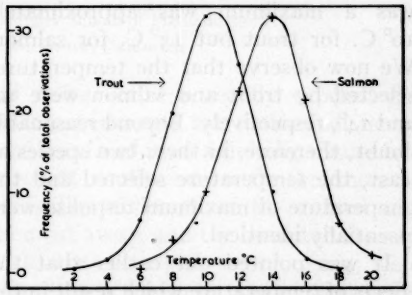


FIG. 4.—The relative frequency with which individual trout (*dots*) and salmon (*crosses*), respectively, were observed in different regions of a temperature gradient. The frequencies are expressed as percentages of the total number of observations made in each case (1,100 approximately for trout and 1,700 for salmon). Each frequency is plotted at the average temperature of the respective region of the gradient.

TABLE 1

STATISTICAL TEST OF THE DATA ON TEMPERATURE SELECTION

| Comparison (1) | Degrees of Freedom (2) | $\chi^2$ (3) | Probability (4) |
|---|---|---|---|
| The 2 halves of Expt. 1 on trout | 3 | 0.86 | >0.61 |
| The 2 halves of Expt. 2 on trout | 6 | 2.56 | 0.77 |
| The 2 halves of Expt. 3 on trout | 5 | 2.85 | 0.58 |
| The 2 halves of Expt. 1 on salmon | 5 | 3.43 | 0.49 |
| The 2 halves of Expt. 2 on salmon | 5 | 1.77 | 0.78 |
| The 2 halves of Expt. 3 on salmon | 4 | 0.41 | >0.80 |
| The 3 trout expts. | 12 | 8.68 | 0.65 |
| The 3 salmon expts. | 12 | 3.21 | 0.98 |
| Combined trout vs. combined salmon | 8 | 32.3 | 0.00008 |

one population of variates. For comparison the value of $\chi^2$ obtained by considering the three sets of data for trout as samples of a single population and similarly the three sets of data for salmon as samples of another population were also computed. These values are given in Table 1, along with those which arose from a comparison of the two halves of the table. It will be noted, first, that the two halves of each experiment do not differ appreciably, nor do the trout and salmon experiments, respectively, differ among themselves. The probability that the data for the trout and salmon are samples of the same population of variates is, however, very low, less than one part in ten thousand. It is reasonable to

conclude on these statistical grounds, therefore, that the distributions of salmon and trout in a temperature gradient are very significantly different.

It was noted above that the temperature at which the response to stimulation was a maximum was approximately 10° C. for trout but 15° C. for salmon. We now observe that the temperatures selected by trout and salmon were 10° and 14°, respectively. Beyond reasonable doubt, therefore, in these two species at least, the temperature selected and the temperature of maximum response were essentially identical.

It was pointed out earlier that the effects of temperature which result in the selection of temperature are effects on a moving organism. It was noted that, as far as the position or location of the organism at any time is concerned, these movements can be completely described in terms of their duration in time, their direction, and the distance traveled in each. There are, in fact, no other terms in which these movements can be accurately described. It follows that if there exists some characteristic of an environment which determines the location of organisms in it, this factor must operate through an effect in the organism on one or more of these attributes of the movements. It has now been found in two species of fish that temperature affects the distance moved in response to a stimulus. Moreover, the temperature at which the response is a maximum is identical with the selected temperature. In the search for the physiological mechanisms of temperature selection, therefore, it may be profitable to entertain the possibility that selection occurs as the immediate consequence of the effect of temperature on the distance moved in response to a stimulus.

## SUMMARY AND CONCLUSIONS

1. It is found that salmon, like trout, respond to an electrical stimulus by making a relatively discrete "dart" forward. The distance traveled in this dart is a function of the strength of the stimulus and of the temperature.

2. The response to the electrical stimulus is found to be a maximum at approximately 15° C. for salmon and 10° C. for trout. The temperature at which the maximum response occurs is probably independent of the strength of the stimulus.

3. When free to move in a horizontal gradient of temperature, salmon select approximately 14° C., while trout select 10° C. after acclimation at approximately 4° C.

4. It is apparent for both species investigated that the temperature selected is identical with the temperature at which a maximum response to stimulation occurs.

5. It is pointed out that selection may be the consequence of the effect of temperature on the distance moved in response to stimulation.

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# FUNCTIONAL ANATOMY OF THE GIANT FIBER SYSTEM OF CALLIANASSA CALIFORNIENSIS

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## INTRODUCTION

PHYSIOLOGICAL studies have been carried out on the giant fibers of relatively few members of the class Crustacea. Early functional observations were made by Johnson (1926) on *Cambarus* and *Palaemonetes;* and Holmes, Pumphrey, and Young (1941) have studied the relation of conduction velocity to myelination in *Leander*. The neurophysiology of *Cambarus* has been comprehensively investigated by Wiersma (1947) and his group. Each new form presents certain advantages for experimental work. *Callianassa californiensis* is recommended for physiological studies by its convenience and by the simplicity of its giant-fiber system. This macrurous anomuran may be collected in large numbers and is easily maintained in laboratory tanks. The nervous system is readily accessible, and its anatomy permits of a wide variety of experimental procedures. Some of the salient features of the giant-fiber system of *C. californiensis* are briefly described in this paper.

## MATERIAL AND METHODS

The *C. californiensis* used in these experiments were collected at Elkhorn Slough, Moss Landing, California. They kept well in tanks of running sea water for periods of 3 weeks or longer. The exoskeleton of this species is thin, almost transparent, and easily cut, so that the nervous system—especially the abdominal nerve cord—is readily exposed. In most experiments the abdominal nerve cord was freed from adjoining tissues but was left continuous with the thoracic cord. The abdomen of the animal was then cut away, and the head and thorax, with the attached nerve cord, were placed in a sea-water bath. Nerves remained in good condition in running sea water for as long as 24 hours. Investigation of the abdominal nerve cord is further facilitated by the fact that the interganglionic connectives between the third and fourth and between the fourth and fifth abdominal ganglia are separate and unfused, so that stimulation or recording may be carried out on either side independently. Each of the abdominal ganglia (with the exception of the first) has three roots, which may be isolated in the manner employed by Wiersma (1947) on the crayfish. Stimuli were delivered through micromanipulated platinum electrodes from an electronic stimulator, which provided three stimuli in any predetermined sequence. Action potentials were picked up by another pair of micromanipulated platinum electrodes and led through a Grass P3 amplifier to a DuMont 208 oscilloscope, the sweep of which was controlled from the stimulating circuit.

## OBSERVATIONS

When the completely isolated abdominal nerve cord of *Callianassa* is stimulated with a series of stimuli of gradually increasing strength and when a relatively

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low threshold is reached, there abruptly appears an action potential of large size (Fig. 1). On further increase of stimulus strength, the spike suddenly doubles in amplitude (Fig. 1). If one of the interganglionic connectives is now cut on either side between the stimulating and the recording electrodes, the double-amplitude spike drops at once to a single-amplitude spike with all-or-none characteristics. Such results are consistent with the presence of two giant fibers, one on each side of the abdominal nerve trunk.

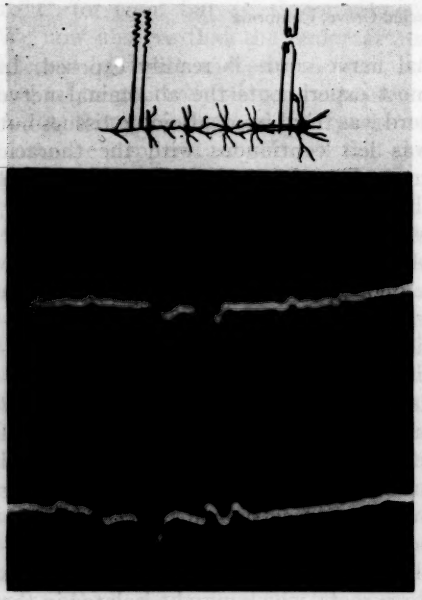

FIG. 1.—Action potentials recorded from the isolated abdominal nerve cord. *Top:* Stimulus reaches the threshold of the giant fiber of one side alone. *Bottom:* Increase of stimulus strength brings in the giant fiber of the opposite side as well. The first deflection in each of the records is that produced by the shock artifact.

Similar stimulation carried out on preparations in which the nervous system is left intact demonstrates further points of interest. In fresh preparations the single-amplitude spike does not occur alone but is followed by a reflected spike (Fig. 2). By simple placement of electrodes it can be ascertained that reflection of the single-amplitude spike occurs from the anterior end. At no time were reflections observed from the caudal end of the nervous system. The reflection may be abolished by cutting one of the circumesophageal commissures or by a midline cut through the brain. The "synapse" may thus be physiologically localized roughly to the supra-esophageal ganglion.

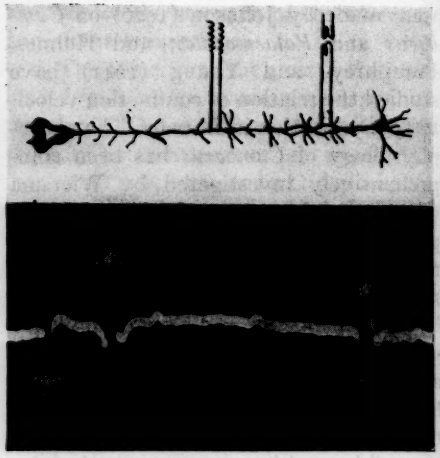

FIG. 2.—Reflection of the giant-fiber spike. The right half of the abdominal chain was stimulated between the fourth and fifth abdominal ganglia. Recording electrodes were between the first and second abdominal ganglia. The action potential here travels up the right giant fiber, reflects in the brain, and travels down the left giant fiber.

This functional connection between the two giant fibers conducts with equal facility in either direction in the same preparation. When the giant fiber in one circumesophageal commissure is stimulated at its entrance into the brain and action potentials are recorded from the thoracic or abdominal cord, the "synaptic delay" is seen to be something less than 0.5 msec. (Fig. 3). Further, the syn-

apse may be readily fatigued by increase of the stimulus frequency—e.g., 10 minutes' stimulation at 90 per second (Fig. 4). By appropriate regulation of stimulus frequency, the synapse may be made to transmit every other preganglionic impulse, every third, or some other ratio of pre- and postganglionic impulses. Facilitation of the synapse by previous conditioning impulses was not obtained, i.e., a condition of the synapse could not be induced in which only the second of a pair of preganglionic impulses was transmitted. It is possible that the optimum frequency of the double stimuli and the optimum spacing between the double shocks were not attained. It can be reported only that many combinations of frequency and spacing were tried and that, of these, none was successful. It was established that the frequency of stimuli necessary to block the synapse was still far short of the frequency which blocked conduction in the giant fibers.

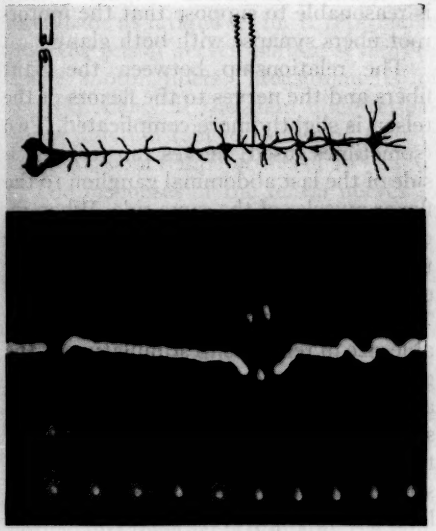

FIG. 3.—Delay of the action potential at the synapse in the brain. The right circumesophageal commissure was stimulated at its entrance into the brain. Recording was from both giants between the second and third abdominal ganglia. A fraction of the delay between the spikes is due to the conduction time from the point of stimulation to the synapse. Time: 1,000 c.p.s.

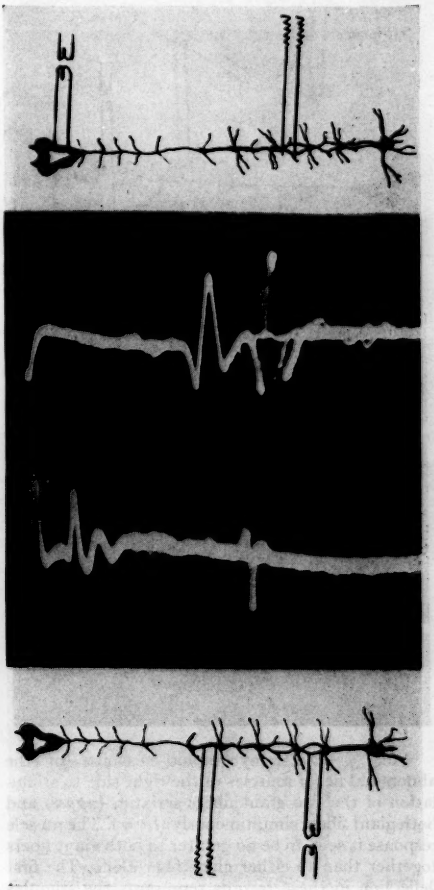

FIG. 4.—Fatigue of the synapse between the giant fibers. In the upper figure the right circumesophageal commissure has been stimulated at a frequency of 90 per second for approximately 10 minutes. Records are taken from both giants between the third and fourth abdominal ganglia. The postganglionic spike is seen to delay in time and finally to drop out. In the lower figure the left half of the abdominal cord was stimulated between the fourth and fifth ganglia, and the recording electrodes were placed across both giants between the first and second ganglia. The postsynaptic (reflected) impulse is seen to drop out all-or-none.

Failure to produce facilitation at the synapse was not, therefore, due to the second of a pair of preganglionic action potentials falling in the refractory period of the first. The functional connection between the giant fibers of *Callianassa* appears, in short, to be very similar to that which exists between the medial giant fibers of the crayfish (Wiersma and Turner, unpublished).

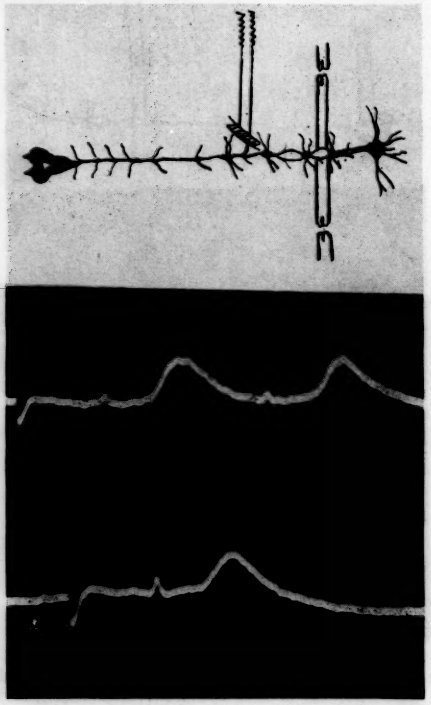

FIG. 5.—Electromyographic response of the abdominal flexor muscles of the right side to stimulation of the two giant fibers seriatim (*upper*) and both giant fibers simultaneously (*lower*). The muscle response is seen to be no greater to both giant fibers together than to either giant fiber alone. The first deflection in these records represents the stimulus artifact. The small deflection just preceding the large muscle responses represents the giant-fiber potential. Note its double amplitude in the lower figure.

On stimulation of the giant fibers of the cord, only a very small outflow may be recorded from the first or second roots of an abdominal ganglion. The third root, however, as in the crayfish, displays action potentials of considerable size. Records from the ventral longitudinal flexor muscles of one side alone indicate a response of equal amplitude upon stimulation of the right giant fiber, the left giant fiber, or both giant fibers simultaneously (Fig. 5). Since there is no evidence to indicate an interconnection of the giant fibers at any of the abdominal ganglia, it is reasonable to suppose that the motor-root fibers synapse with both giants.

The relationship between the giant fibers and the nerves to the flexors of the telson is slightly more complicated. Two (sometimes fused) nerves run from each side of the last abdominal ganglion to the flexor muscles of the same side. When recording electrodes are placed under the telson nerves of one side, the following events are almost invariably recorded: There is an action potential of considerable size in response to stimulation of the giant fiber of the same side. There is an action potential of lesser amplitude in response to stimulation of the giant fiber on the opposite side. If the giant fibers are stimulated by a single shock to the ipsilateral side (e.g., between the third and fourth abdominal ganglia), the larger action potential appears first, followed, at an interval corresponding to the reflection time of the giant fibers, by the smaller action potential, which is set off by the arrival of the reflected giant spike. If the abdominal cord is first stimulated on the contralateral side, the same series of events takes place, except that in this case the smaller of the two spikes appears first, corresponding to the arrival of the contralateral giant impulse before the ipsilateral (in this case reflected) giant spike. When both giants are stimulated simultaneously, the response re-

corded from the telson nerves of one side is identical with, and no greater than, the response recorded after stimulation of the ipsilateral giant fiber alone (Fig. 6).

Neither of the action potentials observed in the telson nerves was all-or-none in its behavior. Often the larger of the two could be made, by increase of the stimulus frequency, to drop out in a series of steps. These steps were inconstant in number from one preparation to another, and in many cases the action potential simply decreased gradually in amplitude as fatigue set in. It is probable that the telson nerves contain several or many motor fibers. Measurements of synaptic delay at the giant-fiber–telson-nerve junction ranged from 0.25 to 0.35 msec. at room temperatures of 20°–22° C.

The responses of the telson nerves to stimulation of the giant fibers would seem to be most simply interpreted by assuming that the ipsilateral giant fiber stimulates all (as a unit term) of a group of fibers, whereas the contralateral giant fiber stimulates only part of a group. Since the stimulation of both giant fibers produces an action potential in the telson nerves of one side identical with that observed by firing the ipsilateral giant, it appears that the group of fibers set off by both giants together is probably the same group as that set off by the ipsilateral giant alone.

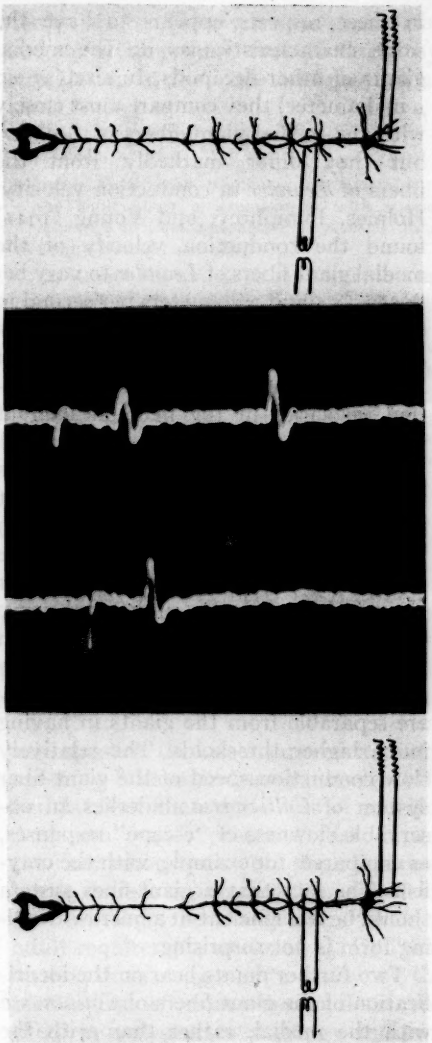

FIG. 6.—Relation of the telson nerves to the giant fibers. Action potentials were recorded from the telson nerves of the right side. In the upper figure the left half of the abdominal nerve cord was stimulated between the fourth and fifth ganglia. The telson nerves are thus stimulated first through the giant fiber of the contralateral side and later by the reflected action potential traveling down the giant of the ipsilateral side. In the lower figure both giants are stimulated simultaneously between the fourth and fifth abdominal ganglia. The response in the telson nerves of the right side is seen to be similar to, and no greater than, that produced by the ipsilateral giant fiber alone.

## DISCUSSION

Despite varying numbers of giant fibers in different decapods, the medial giant fibers of each of the forms thus far studied would appear to be comparable. Thus in *Palaemonetes*, *Crangon*, and *Cambarus* (Johnson, 1924), in *Astacus* (Krieger, 1880), in *Homarus* (Allen, 1894), and in *Leander* (Holmes, 1942) the medial giant fibers show protoplasmic continuity and extend throughout the length of the central nervous system. The single pair of giant fibers of *Callianassa*

in these respects appears to have the same characteristics as do the medial giants of other decapods. In size (35–40 $\mu$ in diameter) they compare most closely with the medial giant fibers of *Leander*, but they differ markedly from the fibers of *Leander* in conduction velocity. Holmes, Pumphrey, and Young (1941) found the conduction velocity of the medial giant fibers of *Leander* to vary between 18.4 and 23.0 meters per second in oil at 17° C. The giant fibers of *Callianassa*, as measured by the method of Katz and Schmitt (1940), had conduction velocities in the neighborhood of 6–7.5 meters per second in sea water at room temperatures of 20°–22° C. In this regard they correspond to the motor-root fibers of *Cambarus*, which, according to Wiersma (1947), have a speed of the order of 7 meters per second. Other groups of fibers in the abdominal nerve cord of *Callianassa* have conduction velocities almost as rapid as that of the giants. Physiologically, however, they are separable from the giants in having much higher thresholds. The relatively slow conduction speed of the giant-fiber system of *Callianassa* underlies an observable slowness of "escape" responses, as compared, for example, with the crayfish. The fact that a giant-fiber system should be less efficient in a burrow-dwelling form is not surprising.

Two further points bear on the identification of the giant fibers of *Callianassa* with the medial, rather than with the lateral, giants of other decapods. One is the fact that, during a large number of observations, no reflections were ever seen from the telson, such as are characteristic of the lateral giant fibers of *Cambarus* (Wiersma, 1947). Second, when the interganglionic connectives of alternate sides are cut or when the interganglionic connectives are cut on alternate sides between every other ganglion, transmission of the giant spikes through the abdominal cord is abolished. Under these conditions, stimulation of the abdominal cord, for example at the first segment, gives rise only to small, diffuse, low-amplitude potentials picked up between the fifth and sixth abdominal ganglia. No large spikes, such as may occur in the lateral giant fibers of *Cambarus* under similar conditions, were ever observed.

Until histological material is carefully examined, the exact anatomical connections between the third roots and the giant fibers and between the telson nerves and the giant fibers remain speculative. The behavior of the motor roots of *Callianassa* suggests a relationship similar to that between the motor roots and the medial giant fibers of *Cambarus*, although a partial fusion of the motor-root fibers of the two sides, as in *Palaemonetes*, *Crangon*, and *Leander*, is possible. To test possible fusion, stimulation of one third root with recording from the third root of the opposite side was tried in a few favorable preparations and resulted in failure to record any transmission. In these instances, however, such failure may possibly be the result of inadequate technique, and, until more of such experiments can be carried out, fusion of the motor roots may be considered admissible.

## SUMMARY

The central nervous system of *C. californiensis* contains a pair of giant fibers 30–40 $\mu$ in diameter, which conduct at a velocity of 6–7.5 meters per second at 20°–22° C. These fibers, comparable to the medial giant fibers of other decapods, make functional contact with each other in the brain. Both giant fibers make equal synaptic connection with motor-root fibers leaving both sides of the abdominal ganglia. The telson nerves are related to the giant fibers in such a way that more telson-nerve fibers are stimulated by the

giant fiber of the ipsilateral side than are stimulated by the giant fiber of the contralateral side. Stimulation of both giant fibers together produces in the telson nerves of one side a response similar to, and no greater than, that produced by stimulation of the ipsilateral giant fiber alone.

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# THE METABOLISM OF STARVED NYMPHS OF THE GRASSHOPPER, CHORTOPHAGA VIRIDIFASCIATA DE GEER


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WIGGLESWORTH (1939) gave a brief review of the chemical changes associated with the starvation of insects. From this review the following generalizations may be made: (1) the available carbohydrates are first consumed; (2) fat is the chief reserve substance to be utilized; and (3) insects differ greatly with respect to their ability to metabolize protein. In some insects a high percentage of the body protein is oxidized before death; while in others there is very little or no utilization of body proteins during complete inanition.

Work done in this laboratory indicates that insects tend to conserve nitrogen during periods when food cannot be ingested. Ludwig (1946) studied the effects of DDT on the metabolism of the Japanese beetle larva and found that, following exposure, the insect survives an average of 5–8 days and uses carbohydrate and fat, but there is no loss of protein. Anderson (1948) observed that during the metamorphosis of the Japanese beetle there is no significant loss of nitrogen, although the change from larva to adult requires 3–4 weeks at 25° C. These observations suggested a study of the metabolism of starvation to determine the principal sources of energy and particularly the extent of nitrogen utilization.

## MATERIAL AND METHODS

Grasshopper nymphs were collected in New York City during the fall and winter of 1948–49. When brought into the laboratory, they were placed in a cage at room temperature and kept abundantly supplied with fresh lettuce leaves.

Chemical studies were made on normal nymphs and on others starved for 1 week or until death. Nymphs used in the starvation experiments were weighed, placed individually in vials, and kept throughout the experiment at 25° C. in a desiccator over a saturated solution of $ZnSO_4$ (relative humidity, 82 per cent). Previous experiments (Ludwig, 1937) had shown that the relative water content of the insect remained constant during exposure to this humidity.

Determinations of reducing substances, expressed as glucose, were made on entire animals by a modified Hagedorn and Jensen procedure (see Ludwig and Rothstein, 1949). Glycogen and fat determinations were made on desiccated insects. The nymphs were weighed and dried to constant weights under vacuum desiccation. Water contents were calculated from the weights before and after complete desiccation. Glycogen was precipitated by a modified Pflüger technique (see Good, Kramer, and Somogyi, 1933). The glycogen was then hydrolyzed to glucose by boiling it in 0.6 N HCl for 3 hours in a water bath. The amount of glucose thus formed was measured by the modified Hagedorn and Jensen procedure, and this value was multiplied by 0.93, the glycogen conversion factor, to obtain milligrams of glycogen. Determinations of fat were made by ether extraction in a Soxhlet apparatus according to the procedure outlined by Ludwig and Rothstein (1949).

TABLE 1

COMPOSITION OF NORMAL NYMPHS

| Substance | No. of Nymphs | Average Weight of Nymphs (Mg.) | Per Cent Weight |
|---|---|---|---|
| Nitrogen | 22 | 90.2 | 2.82* |
| Protein (calc.) | ..... | ..... | 17.63 |
| Fat | 15 | 180.8 | 4.04 |
| Glucose | 40 | 136.5 | 1.31 |
| Glycogen | 29 | 131.5 | 1.71 |
| Water | ..... | ..... | 72.83 |
| Total | 106 | ..... | 97.52 |

* Figure used in the calculation of protein and not included in total.

Nitrogen determinations were made on entire insects by means of the Kjeldahl procedure. Each insect was weighed and then digested in a 100-ml. Kjeldahl flask with concentrated sulphuric acid. The digest was allowed to cool, diluted with 9 volumes of distilled water, alkalized with 30 per cent NaOH, and distilled, the ammonia being received in a 4 per cent boric acid solution. It was then titrated with 0.01 N HCl to match a color standard, with brom cresol green used as the indicator. The weight of the nitrogen was determined by multiplying the number of milliliters of acid required in the titration by 0.1401, the nitrogen conversion factor. Protein was then calculated by multiplying the nitrogen value by 6.25, the protein conversion factor.

The feces eliminated by one group of starving nymphs were collected daily and weighed and were then subjected to nitrogen determinations by the micro-Kjeldahl procedure. The feces eliminated by another group of starving nymphs were collected at the end of the period of complete inanition, weighed, and subjected to nitrogen analysis. Values obtained by these procedures were used in the calculation of the amount of feces egested, the rate of nitrogen excretion, and also the total amount of nitrogen eliminated during starvation.

## OBSERVATIONS

Table 1 contains an analysis of normal nymphs, and Table 2 of nymphs starved for 1 week. During this period there was a relative increase in nitrogen from 2.82 to 3.70 per cent of body-weight. The rel-

ative amount of protein also increased from 17.63 to 23.13 per cent of body-weight. These are only apparent increases, since nitrogen was lost at a much slower rate than some of the other constituents of the insect. There was a re-

week of starvation was the result of a loss of water. During this period almost all the glycogen (97 per cent of the original amount) was utilized. In addition, 67.8 per cent of the glucose and 24.8 per cent of the fat originally present were

TABLE 2

COMPOSITION OF NYMPHS STARVED FOR 1 WEEK

| Substance | No. of Nymphs | Average Weight of Nymphs | | | Per Cent Final Weight |
|---|---|---|---|---|---|
| | | Original Weight (Mg.) | Final Weight (Mg.) | Weight Loss (Mg.) | |
| Nitrogen | 9 | 163.6 | 107.6 | 56.0 | 3.70* |
| Protein (calc.) | ..... | ..... | ..... | ..... | 23.13 |
| Fat | 10 | 215.2 | 168.3 | 46.9 | 3.93 |
| Glucose | 9 | 183.3 | 144.5 | 38.8 | 0.55 |
| Glycogen | 10 | 177.8 | 151.1 | 26.7 | 0.06 |
| Water | ..... | ..... | ..... | ..... | 73.6 |
| Total | 38 | ..... | ..... | ..... | 101.27 |

* Figure used in the calculation of protein and not included in total.

TABLE 3

WEIGHT LOST DURING 1 WEEK OF STARVATION

| Substance | Normal Per Cent | Original Weight (Mg.) | Final Per Cent | Final Weight (Mg.) | Weight Lost (Mg.) | Per Cent Lost |
|---|---|---|---|---|---|---|
| Nymphs | ..... | 185.0 | ..... | 142.9 | 42.1 | 22.8 |
| Water | 72.83 | 134.74 | 73.6 | 105.17 | 29.57 | 21.9 |
| Fat | 4.04 | 7.47 | 3.93 | 5.62 | 1.85 | 24.8 |
| Glucose | 1.31 | 2.42 | 0.55 | 0.79 | 1.63 | 67.8 |
| Glycogen | 1.71 | 3.16 | 0.06 | 0.09 | 3.07 | 97.1 |
| Feces | ..... | ..... | ..... | ..... | 5.27 | ..... |
| Nitrogen | 2.82 | 5.22 | 3.80 | 5.29 | ..... | ..... |
| Total | ..... | ..... | ..... | ..... | 41.39 | ..... |

duction in the amount of carbohydrate, the percentage value of glycogen dropping from 1.71 to 0.06 and of glucose from 1.31 to 0.55. The percentage concentration of water and fat remained approximately constant.

Table 3 shows that, although water was lost at the same rate as body-weight, most of the weight lost during the first

oxidized. The amount of feces eliminated during the first 24 hours averaged 1.65 per cent of the original weight of the nymph. During the remainder of the survival period the average amount of feces eliminated on each day was approximately 0.2 per cent of the original weight of the insect.

Table 4 contains an analysis of

nymphs after complete starvation. The nitrogen content of this group averaged 4.85 and the protein 30.31 per cent of the final body-weight. The relative amounts of glycogen, glucose, and water were approximately the same as at the end of 1 week of inanition. However, the fat content dropped from 4.04 per cent of the original weight to 2.13 per cent of the final weight of the insect.

TABLE 4

COMPOSITION OF NYMPHS STARVED TO DEATH

| Substance | No. of Nymphs | Average Time of Survival (Days) | Average Weight of Nymphs (Mg.) | | | Per Cent Final Weight |
|---|---|---|---|---|---|---|
| | | | Original Weight | Final Weight | Weight Lost | |
| Nitrogen | 30 | 19.0 | 103.6 | 53.2 | 50.4 | 4.85* |
| Protein (calc.) | | | | | | 30.31 |
| Fat | 13 | 15.5 | 153.5 | 87.9 | 65.6 | 2.13 |
| Glucose | 15 | 16.9 | 165.9 | 105.2 | 60.7 | 0.53 |
| Glycogen | 14 | 11.4 | 164.6 | 110.7 | 53.9 | 0.08 |
| Water | | | | | | 72.75 |
| Total | 72 | | | | | 105.80 |

* Figure used in the calculation of protein and not included in total.

TABLE 5

WEIGHT LOST DURING COMPLETE STARVATION

| Substance | Normal Per Cent | Original Weight (Mg.) | Final Per Cent | Final Weight (Mg.) | Weight Lost (Mg.) | Per Cent Lost |
|---|---|---|---|---|---|---|
| Nymphs | | 138.1 | | 82.0 | 56.1 | 40.6 |
| Water | 72.83 | 100.58 | 72.75 | 59.66 | 40.92 | 40.6 |
| Fat | 4.04 | 5.58 | 2.13 | 1.75 | 3.83 | 68.6 |
| Glucose | 1.31 | 1.81 | 0.53 | 0.43 | 1.38 | 76.2 |
| Glycogen | 1.71 | 2.36 | 0.08 | 0.07 | 2.29 | 97.0 |
| Feces | | | | | 6.53 | |
| Nitrogen | 2.82 | 3.89 | 4.85 | 3.98 | | |
| Total | | | | | 54.95 | |

An analysis of the weight lost during complete inanition is given in Table 5. Most of the weight loss (40.9 mg.) was the result of a loss of water. The loss of glycogen remained at 97 per cent, while that of glucose increased to 76.2, and that of fat to 68.6 per cent, of the original amounts present.

Table 6 lists the changes in nitrogen content during complete starvation. The total nitrogen excreted in the feces of starving nymphs amounted to approximately 11 per cent of the original nitrogen present. After the first 1 or 2 days, nitrogen was eliminated at a rather slow, constant rate until the last day of survival, when no measurable amount was excreted. The nitrogen found in the feces added to the nitrogen present in the insect at the time of death amounted to 2.80 per cent of the original weight of the nymphs, as compared with a percentage

of 2.82 in normal nymphs. This observation indicates that practically all the excreted nitrogen was recovered from the feces.

## DISCUSSION

The observation that loss of water accounts for most of the weight lost during starvation is in agreement with the work of Heller (1926) on the adult moth, *Deilephila euphorbiae*. He stated that during the starvation of this insect the loss of water was 66.3 per cent of the original amount and that the dry substance increased from 31.75 to 44.3 per cent.

TABLE 6

NITROGEN CONTENT OF NORMAL NYMPHS AND OF OTHERS AFTER COMPLETE STARVATION

| | No. of Nymphs | Original Weight (Mg.) | Final Weight (Mg.) | Average Nitrogen (Mg.) | Per Cent Nitrogen | Per Cent Protein (Calc.) | Nitrogen Excreted (Mg.) | Nitrogen + Nitrogen Excreted as Per Cent Original Weight |
|---|---|---|---|---|---|---|---|---|
| Controls | 22 | 90.2 | ..... | 2.54 | 2.82 | 17.63 | ..... | ..... |
| Starved | 30 | 103.6 | 53.2 | 2.58 | 4.85 | 30.31 | 0.32 | 2.80 |

According to Wigglesworth (1939), carbohydrate is the main source of energy during the initial phases of starvation in insects. Heller (1926) found very little carbohydrate in the normal adult moth, *D. euphorbiae*, and he gave no figures to show its utilization. Mellanby (1932) observed that in the mealworm, *Tenebrio molitor*, glycogen was used mainly at the beginning of the starvation period, being reduced from 2.04 to 0.68 per cent of the weight of the insect during the first week. In the present experiments 97 per cent of the glycogen was used during the first week, and no additional amount was oxidized during the remainder of the survival period. The figures in Tables 3 and 5 show that most of the glucose also was oxidized in the early phases of starvation.

Fat appears to be the chief reserve substance to be utilized after the store of glycogen has been exhausted. The grasshopper nymph used approximately 25 per cent of its fat during the first week and 68.6 per cent during complete inanition. These figures compare favorably with those obtained by other workers. Slowtzoff (1909) found that, during starvation, the beetle, *Geotrupes stercoralis*, lost 62.8 per cent of its fat. Heller (1926) observed that during complete starvation, which required 9 to 14 days, there was a loss of 70 per cent of the available fat in the moth, *D. euphorbiae;* Mellanby (1932), a loss of approximately 50 per cent during a month's fast in *T. molitor;* and Lafon (1941), a loss of 69 per cent during complete starvation of the fly, *Phormia regina*.

The present experiments show that the grasshopper nymph uses only a small amount of its nitrogen during complete starvation. The rate of nitrogen loss was so slow, compared with the rates at which other constituents were lost, that the percentage of nitrogen increased from a normal of 2.82 to 4.85 during complete inanition. Loss of nitrogen was so small that it could not be demonstrated by multiplying these percentage values by the average original and final weights of all the insects used in the various determinations. This procedure was used for the other constituents (see Table 5). However, a study of the nitrogen metab-

olism in a group of thirty insects showed a loss of approximately 11 per cent of the original amount during the survival period, which averaged 19 days. These observations agree with those of Keller-Kitzinger (1935) on the nitrogen metabolism of the starving honeybee, *Apis mellifica*. She found that during starvation the bee used no body protein, or used it only to a very limited degree. On the other hand, some insects appear to draw more heavily on body protein during starvation. Lafon (1941) stated that he did not calculate with precision the protein used during starvation in the fly, *P. regina*, but it amounted to approximately 16 per cent of the original amount. Slowtzoff (1909) found a loss of 19.32 per cent of the protein in the beetle, *G. stercoralis;* and Heller (1926) observed a loss of 41 per cent of the protein in the moth, *D. euphorbiae*.

The figures contained in Tables 1, 2, and 4 show an increase in the total percentage value of all the determined constituents from 97.52 in normal nymphs to 101.27 in those starved 1 week and to 105.8 in those completely starved. This increase is caused by a change in the value of the calculated protein, which was obtained by multiplying the nitrogen percentage by 6.25. The figure 6.25 is used extensively in calculating protein if the amount of nitrogen is known. It is based on the assumption that nitrogen makes up 16 per cent of the protein molecule. According to Hiller, Plazin, and Van Slyke (1948), the origin of this factor is obscure, but it has been in use for at least seventy-five years. The data contained in this paper indicate that this conversion factor may not be applicable to the proteins in starved grasshoppers, or even in normal insects. The increase in calculated protein may indicate that a change in the amount of protein nitrogen may occur during starvation. One error in the present calculations is the assumption that all the nitrogen determined is protein nitrogen. Hence another possibility is that not all the waste nitrogen is excreted during starvation but that it may be deposited in regions within the insect, as, for example, beneath the exoskeleton. Waste nitrogen in the form of urates or uric acid within the insect is included in the nitrogen determined by these experiments. Its inclusion in the calculation of protein may lead to erroneous results. An attempt to solve this problem by the fractionation of the nitrogenous compounds of normal and starved nymphs is now in progress.

## SUMMARY

1. Determinations were made on the glucose, glycogen, fat, and nitrogen content of normal grasshopper nymphs and of others starved for 1 week, or until death, at 25° C. over a saturated solution of $ZnSO_4$ (relative humidity, 82 per cent).

2. At this humidity, although water is lost at the same rate as body-weight, most of the weight lost was the result of a loss of water.

3. Carbohydrate appears to be the main source of energy during the initial phases of inanition. The grasshopper nymph used 97 per cent of its glycogen during the first week, and no additional amount was oxidized during the remainder of the survival period. Most of the available glucose was oxidized early in starvation, the glucose lost during the first week being 67.8 per cent, and during complete starvation 76.2 per cent, of the original amount.

4. Fat appears to be the chief reserve substance to be oxidized after the glycogen supply has been exhausted. Nymphs lost 25 per cent of their fat during the first week and 68.6 per cent during complete inanition.

5. Nitrogen utilization occurred at a

slow, constant rate, the amount lost during complete starvation being approximately 11 per cent of the total nitrogen. However, because of the rapid loss of body-weight, the percentage of nitrogen increased from 2.82 in normal to 4.85 in completely starved nymphs.

6. The use of the protein conversion factor, 6.25, appears to give erroneous results when used in calculating protein in starved insects. Starvation may lead to an increase in the nitrogen content of the protein, or there may be a storage of waste nitrogen within the insect.

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# EXPERIMENTAL OBSERVATIONS ON COAGULATION OF INSECT HEMOLYMPH


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THE coagulation of insect hemolymph has been studied chiefly from an observational point of view or for the purpose of preventing the coagulation in order to facilitate other studies on the circulating fluid and its constituents. In view of the fact that there is still incomplete agreement on the mechanism of the clotting of mammalian blood, it is not surprising that the analogous phenomenon in insects has not been elucidated, although it may be simpler. Certainly, the explanations for the clotting of mammalian blood have influenced thought on the coagulation of insect hemolymph, although it has long been known that the latter phenomenon cannot be inhibited by oxalates or by citrates. It is quite likely that explanations of hemolymph coagulation in terms of fibrin formation have diverted attention from the study of the physiological

and biochemical aspects of the problem, even though the presence of fibrin has been postulated principally on the basis of visual observation of clotting rather than upon experimental proof of the presence of such a protein. In any case, with a few important exceptions, there has been a greater interest in comparing hemolymph coagulation with vertebrate blood clotting than with the body-fluid coagulation of other invertebrates.

Earlier work on hemolymph coagulation has been reviewed by Yeager *et al.* (1932), and since then most of the work has been done in an effort to find serviceable anticoagulants. Paillot (1923) had reported nucleic acid to be an anticoagulant. Shull *et al.* (1932) demonstrated that acetic acid vapor prevented coagulation; and Shull (1936) reported that the vapors of other fatty acids, such as formic, propionic, butyric, and valeric, had a similar effect. He concluded that the carboxyl radical was responsible for the inhibitory action. Fisher (1936) mentioned hydrocyanic acid, ether, and pyridine as having no inhibiting action. Heat treatment, short of protein precipitation, was found by Yeager *et al.* (1932) to prevent coagulation and to fix hemocytes in their naturally occurring forms. Most of the work on coagulation has been done on the hemolymph of various species of cockroach. Yeager and Knight (1933), however, made observations on forty-seven insect species, among which were found some the hemolymph of which failed to coagulate. In others the hemocytes agglutinated, but the plasma portion was not involved. In a third group of insects both the hemocytes and the plasma took part in the coagulation. These authors noted that the differences were not always sharply defined and that intergradations were encountered. The differences emphasize the lack of similarity between mammalian blood clotting and hemolymph coagulation—a point on which there is general agreement at the present time (Rapp, 1947).

There are numerous differences between the clotting phenomena in mammals and those in insects. Fibrin is much more stable than the hemolymph clot. Calcium plays an important role in the clotting of blood but is not essential in hemolymph coagulation, as is evidenced by the failure of citrates or oxalates to affect the completion of the process. Thrombin and prothrombin are not essential to the coagulation of insect hemolymph, as judged by the failure of the antithrombin hirudin and the antiprothrombin heparin to inhibit coagulation. The absence of both thrombin and fibrinogen is indicated by the experiment of Babers (1938), in which hemolymph, from larvae of *Prodenia eridania*, added to thrombin or fibrinogen from normal horse serum, failed to produce clotting.

The coagulation phenomenon in insects is probably nothing more than an incidental characteristic of the protein-carbohydrate complex in the fluid rather than a mechanism which has survival value in an evolutionary sense or physiologic value in reducing hemorrhage. The absence of coagulability in the hemolymph of some insects is one reason for this point of view, and also some insects are reported to have a hemolymph pressure less than atmospheric pressure, so that no hemorrhage occurs upon wounding. It was noted by Beard (1949) that Japanese beetle grubs, punctured with a needle, bled just as much when the issuing hemolymph was allowed to coagulate as when the hemolymph was promptly removed. Moreover, physiologic recovery and relacement of body fluid was reasonably prompt after hemorrhage. If wounding should occur in an appendage so as to permit a very slow flow of hemolymph, coagulation might be a

physiologic advantage; but, if wounding should occur in the general body regions in association with a positive hemolymph pressure, there is likely to be a sudden gush of fluid (more comparable to arterial hemorrhage in mammals), and coagulation is of little value, in spite of its rapidity. In cases in which "reflex bleeding" involves the hemolymph and not glandular secretion, this phenomenon is another indication that the loss of hemolymph may not be of serious consequence.

The observations reported below are insufficient to provide an adequate explanation of hemolymph coagulation, but they are presented in the hope that some thought can be stimulated in this direction and that the details will find usefulness in related studies. Most of the observations were made on the third instar larva of the Japanese beetle (*Popillia japonica* Newm.), but the larva of the wax moth (*Galleria mellonella* L.) was also used.

### COAGULATION OF THE HEMOLYMPH FROM LARVAE OF *Popillia japonica*

According to the classification of Yeager and Knight (1933), the coagulation of the hemolymph of the Japanese beetle grub is of the third type, in which both the hemocytes and the plasma take part. When a drop of hemolymph from the Japanese beetle grub is exposed to air, it coagulates in a matter of seconds by gelation of the plasma, which entraps most of the hemocytes. The coagulum can be gathered into a viscous drop with a needle, leaving a small volume of more fluid "serum" containing the scattered cells which did not become enmeshed in the coagulum. The cells in the coagulum appear to be passively held by the clot, as they are randomly spaced with no agglutination. Although some cells have short "pseudopodia," these do not anastomose. Phase-contrast microscopy discloses a few cells with longer, threadlike pseudopodia, but these are much less prominent than those present in the hemolymph of the cockroach. No structure is evident in the intercellular matrix, which appears hyaline except for some lipomicrons or other scattered particulate matter. At the periphery of the coagulum there is a "membrane formation" in which the cells show a more definite orientation, but this is undoubtedly a phase-boundary phenomenon rather than an active agglutination of these cells. The proportion of the hemolymph taking part in the coagulation is variable, ranging from about 85 to about 96 per cent by weight, the remainder being left as "serum." Haber (1926) stated that with the hemolymph of the cockroach, *Blattella germanica*, the "fibrin" clot, as in mammalian blood, contracts and squeezes out the serum. In contrast to this, the coagulum of the Japanese beetle grub does not necessarily become reduced in size upon standing, although some syneresis is likely to occur. If bacterial action is permitted, however, complete liquefaction results. Also, if coagulated hemolymph is stirred, viscosity is reduced to the point of liquefaction, thus indicating one instance of the instability of the clot. This is scarcely comparable to defibrinated blood, in which fibrin is produced but is removed as it is formed. Clotting occurs in an atmosphere of carbon dioxide as readily as in the open air.

### COAGULATION OF THE HEMOLYMPH FROM LARVAE OF *Galleria mellonella*

The coagulation of the hemolymph from wax-moth larvae, in contrast to that of the Japanese beetle grub, characteristically involves the hemocytes alone, although all three hemolymph types of the Yeager and Knight classification may be found in different specimens of the one

insect. At times in the absence of diffusion currents or agitation, there is no cellular agglutination, and the plasma remains fluid. At other times the plasma may become more or less viscous, even coagulating as completely as that from Japanese beetle grubs. More commonly, the cells agglutinate to form what appears grossly as a flocculant precipitate more or less combined in irregular masses, the plasma remaining fluid. These differences in response cannot at present be associated with any event or stage of development of the organism. Since they may occur in different individuals from the same laboratory culture, seasonal variations are not involved, as Yeager and Knight (1933) observed in the field cricket. Certainly, these different responses make it difficult to interpret the effect of treatment.

### MODIFICATION OF HEMOLYMPH CLOTTING

Experimental studies on coagulation customarily involve treatment to interfere with the process or some phase of it. With the clotting of mammalian blood, fibrin formation is a convenient end-point for judging the effect. With coagulation of insect hemolymph, the clot is less definite, and inhibition may be of different degrees or may be accompanied by other phenomena. With hemolymph of the Japanese beetle grub, for example, treatment may modify coagulation by permitting an obvious increase in viscosity, but not to the extent of "membrane formation." There may be an induced coagulation caused by precipitation of the formed elements and the plasma which is not comparable to normal coagulation. (The heavy metals characteristically do this.) The formed elements alone may be induced to clump and form a precipitate in the fluid plasma. Or there may be a real inhibition, without complications, in which the plasma remains fluid and the formed elements essentially remain dispersed. Although the last-mentioned response is the desired one in searching for an inhibiting agent, the several responses may not be sharply defined but may grade into each other to make interpretation difficult.

### EFFECT OF HEAT

Yeager *et al.* (1932) observed that immersion of insects in water heated to about 60° C. killed the insects but prevented coagulation of the hemolymph. Japanese beetle grubs heated in water to this temperature similarly yield hemolymph which does not coagulate. Such hemolymph does not contain an anticoagulant (or too little to be effective); for, if a drop of untreated hemolymph is added to heat-fixed hemolymph, plasma gelation takes place. Superimposed upon this, however, is a tendency for the hemocytes to agglutinate. *Popillia* hemolymph added to heat-treated *Galleria* hemolymph responded in the same way, i.e., the plasma gelled and the cells agglutinated. It appears essential for the hemolymph to be within the insect for heat treatment to be effective. If a piece of aluminum foil is floated on water heated to 60° and a drop of hemolymph is allowed to fall from a needle-pricked grub, the hemolymph does coagulate. In this instance it may be that the process of coagulation is initiated so fast that it is under way before the heat-sensitive system is inactivated. On the other hand, the heat-sensitive system may be associated with body tissues other than the fluid which exudes from a wound. *Ex vivo* exposure of hemolymph to temperatures almost high enough to precipitate some proteins still permits coagulation.

Although Muttkowski (1924) claimed that gelatin formed a part of the coagulum in insect hemolymph, the gel state of the hemolymph from Japanese beetle

grubs is not gelatin if by this term is meant a material which reversibly passes from a gel to a sol state upon heating. Such an effect of heat has not been demonstrated.

### EFFECT OF FREEZING

Coagulation may also be inhibited by freezing. If a grub is frozen and allowed to thaw, the hemolymph drawn from it will not gelatinate. In contrast to the effect of heat, if a drop of hemolymph is allowed to fall on a cold plate so that freezing occurs promptly, this drop, upon thawing, does not coagulate, nor do the hemocytes agglutinate. If to a drop of previously frozen hemolymph is added a drop of untreated hemolymph, the response is variable; with most trials coagulation occurs, but with some it does not. Interpretation of this is difficult, but it implies the possible presence of an anticoagulant in the cold-treated hemolymph. The microscopic appearance of the cold-treated hemolymph is somewhat more granular than that of heat-treated hemolymph. This would be expected if cytolysis or cellular fragmentation was induced by freezing and thawing. A variable amount of cellular fragmentation might explain the variable presence of the anticoagulant in cold-treated hemolymph. This is supported by the observation that the viscosity of the coagulum is definitely reduced when untreated hemolymph is added to saline extracts of macerated body-wall tissue.

### EFFECT OF ULTRASONIC WAVES

Under certain conditions, hemolymph exposed to ultrasonic waves will fail to coagulate.[1] If Japanese beetle grubs are placed in water in a suitable container and exposed to 400 kilocycles at a certain range of power, hemolymph drawn from them will not coagulate. The power required varies with the type of container used for exposure, but relatively low power is effective. Higher powers rupture the gut or cause other damage that results in death, but there is a reasonable range of sublethal exposures which will effectively inhibit coagulation. The time of exposure to ultrasonic waves appears to be relatively unimportant. Even momentary exposure to waves of effective power is adequate, and longer exposure does not seem to increase mortality if the power is not too great. If treated grubs are held for observation, hemolymph drawn from them some time after treatment will coagulate. This is the only instance so far noted of a reversible inhibitory effect on coagulation. The time required for a grub to recover the coagulability of its hemolymph is highly variable, ranging from about 10 minutes to 3 hours. Grubs receiving lethal exposures may not die for 24 hours or more, but these grubs do not recover the coagulability of their hemolymph. It is doubtful, however, that this is the lethal action. The recovery of coagulability is limited to the hemolymph *in vivo*. Drawn hemolymph, inhibited by ultrasonic treatment and placed in a moist chamber, remains in an uncoagulated state. It might be mentioned, however, that melanization occurs, as in untreated hemolymph.

Hemolymph from grubs treated with ultrasonic waves does not possess anticoagulant properties; for, if freshly drawn hemolymph from untreated grubs is added to it in equal amounts, normal coagulation takes place.

If hemolymph is allowed to drop from a punctured grub onto a metal foil in the zone of impact of ultrasonic waves, coag-

[1] Most of the observations on effects of ultrasonic waves were made with the co-operation of Dr. Raymond Wallace, of the University of Connecticut, to whom the thanks of the writer are due. A few of the observations were made through the kindness of Mr. S. I. Ward, of the Crystal Research Laboratories, Hartford, Connecticut.

ulation occurs in a normal manner. Greater power will precipitate proteins but will not otherwise affect coagulation. Again, a possible relationship between the other body tissues and the circulating fluid is suggested, which becomes more evident in view of the following observations. If the drop of hemolymph exposed in contact with the metal foil in the zone of impact is suspended from the punctured grub instead of being allowed to fall from it, coagulation does not take place. Moreover, it is possible to inhibit coagulation if exposure of the hemolymph (*ex vivo*) to ultrasonic waves is made in the presence of tissue fragments, such as bits of the body wall. This is not always successful, indicating possible qualitive differences in tissues in their ability to mediate inhibition. If a drop of hemolymph from an exposed grub is placed on metal foil in the zone of impact and a drop of hemolymph from an unexposed grub is added to it, coagulation does not occur. In these several cases the modified hemolymph, the tissue fragments, or the body of the grub contributed something necessary for the ultrasonic waves to be effective. Or, expressed as another possibility, the ultrasonic waves released something from these tissues which served to inhibit coagulation.

The effect of ultrasonic waves on hemolymph of the wax-moth larva is possibly subject to less definite interpretation, but, in general, *in vivo* exposure results in the hemolymph's losing any tendency to increase in viscosity upon exposure to air and the cells' losing their tendency to clump together. Again, *ex vivo* exposure of the hemolymph to ultrasonic waves fails to modify the coagulation. If a drop of hemolymph from a treated wax-moth larva is placed on metal foil in the zone of impact and a drop of hemolymph from an untreated beetle grub is allowed to fall in it, normal gelation of the plasma takes place. This indicates that hemolymph from treated wax-moth larvae, in contrast to that from treated grubs, fails to mediate the inhibition of coagulation of grub hemolymph upon exposure to ultrasonic waves. This is one indication that the fundamental mechanism of coagulation is different in the two insects and that the coagulation of grub hemolymph is not just a more complete form of the coagulation as observed in wax-moth larval hemolymph. This does not prove, however, that the plasma gelation of *Galleria* hemolymph, when it occurs, differs fundamentally from the coagulation of *Popillia* hemolymph.

The mechanism of inhibition by ultrasonic waves is not known. The action is not too drastic, because recovery occurs in such a short time. There is no obvious change in the number or form of the hemocytes, but the hemolymph from treated grubs appears to have more abundant lipomicrons, at times enough to give the fluid a definite turbidity. In view of the possibility that some of the body fat was emulsified, thereby changing the physical state of the fluid, some material, extracted by ether from grubs, was placed on foil in the field of the ultrasonic waves, and a drop of hemolymph was added. This coagulated normally. A bit of vegetable fat similarly failed to mediate inhibition by ultrasonic waves. In both cases emulsification was poor, and these negative tests possibly have little meaning, since the natural complex was not closely simulated. It is unlikely that the ultrasonic treatment is effective because of the heat produced. The effect may be observed when the exposure is too short for much heat to be generated, and also the heat required to inhibit coagulation is fatal to the insects. Although the presence of

some particulate matter in the hemolymph from treated grubs indicates possible breakdown of cells, this cannot be of long-lasting effect, if it does happen, in view of the rapid recovery. The possible oxidizing effect of the ultrasonic treatment is difficult to evaluate, but treated hemolymph fails to form a ropy precipitate with acetic acid, as does hemolymph oxidized with potassium permanganate.

### EFFECT OF CHEMICAL SUBSTANCES

Chemical interference with the coagulation phenomenon is difficult to study with precision. The variation in response has already been indicated, and difficulties of technique impose limitations on the quantitative estimate of effect. It cannot be assumed that substances injected into the body cavity of an insect will circulate freely, to mix uniformly with the hemolymph. For instance, it is known that certain dyes are preferentially sorbed on the body wall at the point of injection, even in cases where the dyes freely soluble in the hemolymph *ex vivo*. Also cellular precipitation or agglutination within the body might not be evident from observation of a sample of hemolymph drawn from the insect. The rapidity of coagulation is a handicap in adding chemical substances *in vitro*. Clotting may be initiated before the hemolymph is thoroughly mixed with the test compound, and it is possible for excessive stirring to liquefy the formed coagulum. Moreover, because of the rapidity of the phenomenon, it is not feasible to measure the quantity of hemolymph used. Unless otherwise indicated, the chemical effects mentioned below are based on observations following the addition of a drop of hemolymph to a drop of the solution being studied, which is promptly stirred, but with the stirring kept at a minimum. When the concentration of the substance is indicated, it refers to the solution as used and not to an estimate of the resultant concentration after admixture with hemolymph.

It has already been noted that the blood anticoagulants heparin, hirudin, citrates, and oxalates have no effect upon the coagulation of hemolymph from Japanese beetle grubs. The same can be said of dicoumarol; but, in view of what is known in regard to how dicoumarol exerts its effect, it would not be expected to act *in vitro*. However, when dicoumarol was injected enterally in Japanese beetle or wax-moth larvae in single or repeated dosages, no effect on coagulation was observed.

Inhibition of coagulation by heat indicates an essential heat-labile substance, and an enzyme system is suggested. The possibility that an enzyme may affect coagulation has not gone without mention in the literature, but no demonstrations of such behavior have been made. Muttkowski (1924) inferred from the presence of "fibrin" that thrombin and thrombokinase must be present. However, he and also Shull *et al.* (1932) observed that, in insects killed by cyanide fumes, hemolymph coagulation was not affected. Hemolymph from Japanese beetle grubs mixed with calcium cyanide coagulates normally. Sodium azide also has no effect on hemolymph coagulation. Although individual oxidizing and reducing agents and metal precipitants have some effect on coagulation, none as a group could be said to inhibit. From this it can be concluded that enzyme systems sensitive to these reagents are not concerned with the gelation phenomenon. Certain of the β-chloroethyl amines inhibited coagulation in *Galleria* hemolymph but not in *Popillia* hemolymph. This would indicate a possible cytotoxic action rather than general enzyme inactivation.

If hemolymph is added to a series of phosphate buffers (Sörenson), coagula-

tion occurs throughout the range of pH from about 5 to 8. In McIlvaine buffers, on the other hand, a graded response is observed. At about pH 5 and below, coagulation does not occur; but, with increasing pH, coagulation becomes more pronounced. In this case the inhibition is due to the citric acid, which prevents coagulation when used alone. It is doubtful whether low pH alone has much direct effect, as solutions have been noted with a pH as low as 3.8 which did not inhibit coagulation. The fatty acids mentioned in an early paragraph are not the only organic acids which will prevent coagulation, and concentrations of HCl as low as N/160 have been observed to prevent gelation in *Popillia* hemolymph, although an increase in viscosity was evident.

Coagulation is inhibited by ascorbic acid in concentrations approximating 0.01 M. Such inhibition is not strictly irreversible because, if hemolymph from beetle grubs is added, drop by drop, to a solution of ascorbic acid, it remains fluid at first. There comes a point, however, when the mixture becomes very viscous, and the addition of another drop or so of hemolymph results in a typical coagulation. Not only are the last few drops involved in the coagulum, but almost the entire mass gelatinates. This indicates that the concentration of ascorbic acid becomes so low that it not only fails to inhibit the hemolymph added but also no longer prevents the coagulation of that inhibited at first. A different result is observed if hemolymph inhibited by ascorbic acid is centrifuged. In this case the plasma portion behaves just as water does, in that a fresh drop of hemolymph added to it coagulates normally. The cellular portion of the centrifugate contains the inhibitor, as evidenced by its preventing coagulation in a fresh drop of hemolymph. Although the failure of the plasma portion to enter into the clot of the hemolymph added to it might suggest a greater role of the cells in the coagulation phenomenon, this conclusion is not warranted without knowing the action of ascorbic acid on the plasma. From these observations it is evident that ascorbic acid does not destroy the coagulable portion of the plasma, although it may bind it in some way to the cells so that it can be removed by centrifuging.

It was observed that surface active agents (such as polyoxyethylene sorbitol oleate-laurate in a 0.1 per cent solution) inhibited coagulation in hemolymph from both Japanese beetle grubs and wax-moth larvae. With the thought that the action of this material served to disperse the cellular constituents of the hemolymph, thus preventing their agglutination, it was considered that mere dilution of the hemolymph *in vivo* might accomplish the same purpose. Accordingly, Ringer's solution was injected into the body cavities of the two insects. Following the injection of rather large amounts, the hemolymph drawn from wax-moth larvae did fail to coagulate. That from Japanese beetle grubs, however, coagulated normally, in spite of excessive dilution. Oddly enough, diluted hemolymph from wax-moth larvae injected into Japanese beetle grubs interfered with the coagulation of the grub hemolymph.

Among a large number of compounds tested for effects on coagulation, those listed in Table 1 modified coagulation in some way in one insect or the other. In addition, there were a few other compounds which had some inhibiting action, but because of inconsistent results they are not included here. Although some of the compounds tested were chosen at random, most of them were selected because they represented a type of action

such as metal precipitation, enzyme inactivation, oxidation, or reduction or were cytotoxic or hemorrhagic.

It is apparent that, although some of the substances modify both types of coagulation, others inhibit one but not the other. It will be noted that the effective concentrations, particularly for preventing gelation in the grub hemolymph, are high. This might be explained by the speed of coagulation and the viscosity of the medium, requiring a higher concentration than might be necessary if diffusion were more prompt. A disturbing aspect of this is that citric, malic, and oxalic acids and oxyquinoline sulfate were effective at one concentration but not at either higher or lower concentrations. This suggests a pH effect, but from what has been said before, the evidence seems to be somewhat conflicting in this regard. Possibly some of the ineffective compounds might have inhibited coagulation if they had been tested at different concentrations. It is also likely that the apparent effectiveness of some compounds at one time and not at others was due to the use of different concentrations in terms of the body fluids. Among the compounds listed in Table 1 are a few metal precipitants. Other reagents having the same action, however, were ineffective, but possibly their failure was due to differences in solubility. Undoubtedly, there are many other compounds than those indicated which will be found to modify coagulation, if a further search is made.

The gelation phenomenon is most suggestive of some type of polymerization induced when hemolymph exudes from a wound. In some respects the coagulum resembles an acid mucopolysaccharide, and an adaptation of Hale's (1946) test for this class of compounds yields a positive reaction with hemolymph. Contrary to expectation, the response is more definite with *Galleria* hemolymph than with *Popillia*. It is noteworthy, however, that hyaluronidase will prevent gelation of *Popillia* hemolymph but has no effect on the formed clot. With this material, too, the quantity required to prevent coagulation is large in comparison with that which will reduce the viscosity of hyaluronic acid. The viscosity of hyaluronic acid (in saline extract of human umbilical cord) is not appreciably affected by ultrasonic waves. Obviously, then, the

TABLE 1

COMPOUNDS MODIFYING HEMOLYMPH COAGULATION

| Compound | Molar Concentration | Effect on Gelation (Popillia) | Effect on Cell Agglutination (Galleria) |
|---|---|---|---|
| Citric acid | 1 | +* | −* |
| | 0.1 | − | − |
| | 0.01 | + | − |
| Malic acid | 1 | + | −p |
| | 0.1 | − | −p |
| | 0.01 | + | −p |
| Oxalic acid | 1 | +p | −p |
| | 0.1 | −p | − |
| | 0.01 | + | + |
| Oxyquinoline sulfate | 1 | +p | P |
| | 0.1 | − | −p |
| | 0.01 | + | + |
| Potassium ethyl xanthate | 1 | −p | −p |
| | 0.1 | + | + |
| Chloral hydrate | 1 | −p | P |
| | 0.1 | + | p |
| | 0.01 | + | + |
| Sodium azide | 1 | + | − |
| Salicylaldoxime | 0.1 | + | + |
| | 0.1 | −P | −P |
| Potassium permanganate | 0.01 | Dissolves | + |
| Methylene blue | 0.1 | − | + |
| Sodium hydrosulfite† | 1 | − | − |
| | 0.01 | + | − |

*+ = permits; − = prevents; p = light precipitate; P = heavy precipitate.

† Indicated by Hensill (1948) to be an inhibiter of decapod blood.

factors which prevent "polymerization" of hemolymph are not necessarily the same as those which depolymerize or hydrolyze the acid mucopolysaccharide. It may be significant, however, that a mucin precipitated by acetic acid resembles the precipitate formed when *Popillia* hemolymph is treated with acetic acid after oxidation with potassium permanganate. This precipitate has not been observed in *Galleria* hemolymph, either before or after such oxidation.

## DISCUSSION

The observations and experiments reported above are insufficient to permit an explanation of the coagulation phenomenon in insect hemolymph, but a few conclusions may be drawn. It seems clear that cellular agglutination and plasma gelation, as represented in the two ininsects studied, are separate and distinct phenomena which can occur independently or together in the same insect or in different insects. In the presence of the plasma coagulum, agglutination of hemocytes may not normally occur but can be induced, as by adding heat-treated hemolymph to untreated hemolymph. If plasma coagulation is inhibited, cell agglutination is more likely to occur. Although the cell pseudopodia may facilitate the adhesion of cells, they alone do not appear to be responsible for agglutination, and inhibitors of this aspect of coagulation presumably modify the cells or their membranes in such a way as to prevent their clumping. The data of Yeager and Knight (1933) strongly suggest that the presence or absence of cell agglutination is correlated with the relative numbers of circulating hemocytes. This has some support in the observation that dilution alone may prevent clumping. The plasma clot is relatively stable, once it is initiated; but its precursors or catalysts, as the case may be, are sensitive to heat, freezing, ultrasonic waves, and a number of chemical agents. Because of the diverse nature of the various inhibitors, it is likely that the gelation is not a simple change in state or an autonomous polymerization but rather some type of catenary system. There is no reason to believe, however, that this resembles the chain of events known to occur in the clotting of mammalian blood.

## SUMMARY

Although early attempts to explain the mechanism of coagulation of insect hemolymph were in terms of the clotting phenomenon in mammalian blood, it now seems unlikely that the two processes are comparable.

Hemolymph withdrawn from Japanese beetle grubs (larvae of *P. japonica*) coagulates by a gelation of the plasma. The hemocytes do not agglutinate except under special conditions.

Hemolymph withdrawn from larvae of the wax moth (*G. mellonella*) characteristically coagulates by an agglutination of the hemocytes. At times, however, the plasma gelatinates, and at other times there is neither hemocyte agglutination nor plasma clotting.

The well-known method of heat treatment for preventing hemolymph coagulation is effective only when exposure to heat is made *in vivo*.

Freezing of hemolymph either *in vivo* or *ex vivo* prevents coagulation.

Hemolymph from *Popillia* or *Galleria* larvae exposed to certain sublethal powers of ultrasonic waves fails to coagulate. This effect on coagulability is only temporary. Hemolymph of *Popillia* larvae exposed *ex vivo* to ultrasonic waves coagulates normally unless the hemolymph is in contact with the body wall of

the grub, certain tissue fragments, or hemolymph from previously exposed grubs. Hemolymph from treated *Galleria* larvae will not mediate the action of ultrasonic waves in *Popillia* hemolymph exposed *ex vivo*. This indicates a fundamental difference in the hemolymph from the two species of insect.

Several chemical substances interfere with coagulation, but these are not necessarily the same for the two species of insect studied. No particular type of action (such as oxidation, reduction, metal precipitation, etc.) was common to several compounds which were observed to modify the coagulation phenomenon.

Cell agglutination and plasma clotting appear to be physiologically distinct phenomena. The former is presumed to be associated with properties of the cell membrane manifested in hemolymph containing a relatively large number of cells. Although hemocytes may be involved in plasma clotting, their role is not known at present. The precursors or catalysts of the plasma clot are much more sensitive to physical and chemical action than is the clot itself.

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# STUDIES ON THE RESISTANCE OF INSECTS TO INSECTICIDES
## I. CHOLINESTERASE IN HOUSE FLIES (MUSCA DOMESTICA L.) RESISTANT TO DDT

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Bureau of Entomology and Plant Quarantine

THE fundamental mechanism by which insecticides produce their effects is still a matter of conjecture. For some time, however, the evidence has been mounting that many, if not all, poisons act by attacking one or more of the essential intracellular enzyme systems (Hurst, 1945; Dixon and Needham, 1946; Green, 1946). The acetylcholine-splitting enzymes, generally grouped under the term "cholinesterases" because of their importance in the transmission of nerve impulses and their universal presence in the insect tissues tested (Bacq, 1935; Corteggiani and Serfaty, 1939; Means, 1942), were early suspects in studies on the physiological action of DDT. No change in the cholinesterase activity of DDT-poisoned insects was observed by Lauger and his coworkers (Lauger *et al.*, 1946), or by Truhaut and Vincent (1947, 1948). However, Tobias, Kollros, and Savit (1946) reported that acetylcholine increased in DDT-poisoned house flies and cockroaches.

The specificity of the acetylcholine-splitting enzymes has been the subject of considerable controversy. It now seems clear that several different esterases or groups of esterases have been given the name "cholinesterase"; and there is abundant evidence that, in accordance with custom, at least one of the esterases can be correctly called "acetylcholinesterase" (Augustinsson and Nachmansohn, 1949). Whether or not the acetylcholine esterase found in different species is the same enzyme remains to be determined.

The kinetics of cholinesterase from several species have been studied by Alles and Hawes (1940), Clark *et al.* (1938), Glick (1941), Nachmansohn and Rothenberg (1945), and others.

Richards and Cutkomp (1945), using the brains of worker honeybees and the nerve cords of cockroaches, found that acetyl-$\beta$-methylcholine was hydrolyzed 2.7–3.0 times faster than acetylcholine was. Tobias *et al.* (1946) also found that acetyl-$\beta$-methylcholine was hydrolyzed faster than acetylcholine by cholinesterase from the roach nerve cord. Tahmisian (1943) found that the optimum pH for the enzyme in grasshopper eggs is 8.5 and the optimum temperature about 40° C. These values are similar to those reported by Glick (1941) for the enzyme from horse serum.

Chadwick and Hill (1947) injected into cockroaches several materials that exhibit anticholinesterase activity *in vitro*. The cholinesterase activity *in vivo* was lowered, and in one case the degree of inhibition followed closely the percentage of mortality.

Research on the development of resistance to insecticides by insects has been in progress in this bureau for some time. Babers (1949) has recently published a comprehensive review on the subject.

It is the purpose of this paper to present data indicating a difference in cho-

linesterase activity for at least 5 days after emergence between normal house flies and those resistant to DDT and to describe the properties of the cholinesterase found in the house fly (*Musca domestica* L.).

## MATERIAL AND METHODS

House flies were reared in the laboratory on standard larval medium (Anon., 1949), and the adults were fed the gelatin-milk formula[1] in use at the laboratory of the Bureau of Entomology and Plant Quarantine, at Orlando, Florida. The colony of flies resistant to DDT was started from pupae obtained from that laboratory (Lindquist and Wilson, 1948). Resistance to DDT was maintained by exposing succeeding generations to such doses of DDT that about 90 per cent were killed and by using eggs from the survivors to carry on the colony. The normal insects were from the bureau's colony at the Beltsville, Maryland, laboratory and had never been exposed to DDT.

Adult house flies were separated according to sex, and the heads were removed while the flies were under carbon dioxide anesthesia. After weighing, the entire heads, usually from 20 to 100 in number, were ground in a Potter-Elvehjem type homogenizer and diluted to the desired volume with 30 per cent glycerol. If a few drops of toluene were added, the brei could be kept in the refrigerator for several weeks at least, with negligible loss of activity.

Cholinesterase was determined either by a modified Alles and Hawes (1940) titration method or by a Warburg technique similar to that described by Ammon (1933).

[1] 20 gm. of Bacto Agar, 40 gm. of dehydrated banana powder, 300 gm. of sugar, 150 gm. of powdered whole milk, 50 gm. of gelatin, 1 ml. of 40 per cent formaldehyde, and 3 liters of water.

In the titration method, to 0.5-ml. aliquots of brei, equivalent to 50 mg. of tissue, and acetylcholine bromide (final contrentration 0.045 M) in a total volume of 3 ml. was added 0.02 N sodium hydroxide at such a rate that the pH was maintained within $\pm 0.2$. The pH was determined with a small glass electrode. The temperature was maintained at $24° \pm 1°$. The volume of alkali used was then plotted against time. Twenty minutes was considered the "standard" interval.

In the Warburg technique, 0.5-ml. aliquots of brei equivalent to 10 mg. of tissue and 2.0 ml. of Ringer bicarbonate buffer ($MgCl_2$, 0.04 M; NaCl, 0.15 M; $NaHCO_3$, 0.031 M final concentration) were placed in a standard Warburg flask. One-half milliliter of acetylcholine bromide solution (final concentration 0.045 M) was placed in the side arm, and the flasks were swept with 95:5 mixture of oxygen and carbon dioxide and immersed in the bath at 37°. After equilibration for 30 minutes, the substrate was mixed with the brei and buffer, and the evolution of carbon dioxide was determined in the usual manner. Fifteen minutes was the usual time interval. The pH at the start of the run was 7.24 and at the end of the determination was about 7.2.

## RESULTS

### CHOLINESTERASE AND RESISTANCE TO DDT

Acetylcholine esterase activity in the strain of flies resistant to DDT was consistently lower than that in the normal colony until the fifth day after emergence. From that time on, there was no consistency in the results. The male insects, on a weight basis, showed much greater activity than did the females, except in newly emerged flies, in which the activity was equal. In each case, therefore, corresponding sexes should be compared. The males are generally much

shorter lived than the females and for that reason were seldom available after the eighth day. The results of these experiments are shown in Table 1.

### SPECIFICITY OF THE ENZYME

The comparative specificity of the enzyme for several choline esters was determined. The results are shown in Table 2.

TABLE 1*

CHOLINESTERASE ACTIVITY OF NORMAL FLIES AND OF THOSE RESISTANT TO DDT

| Age (Days) | Normal Flies | | Resistant Flies | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Just emerged | 0.187 | 0.187 | 0.130 | 0.142 |
| 1 | 0.475 | 0.350 | 0.307 | 0.212 |
| 2 | 0.437 | 0.207 | 0.412 | 0.187 |
| 3 | 0.545 | 0.287 | 0.382 | 0.187 |
| 4 | 0.512 | 0.400 | 0.487 | 0.200 |
| 5 | 0.612 | 0.312 | 0.325 | 0.207 |
| 6 | 0.427 | 0.225 | 0.525 | 0.482 |
| 7 | 0.645 | 0.357 | 0.512 | 0.350 |
| 8 | 0.502 | 0.307 | 0.717 | 0.337 |
| 9 | ..... | 0.262 | 0.537 | 0.362 |
| 10 | ..... | 0.267 | ..... | 0.255 |
| 11 | ..... | 0.317 | ..... | 0.287 |

* Rates are in milliliters of 0.02 N sodium hydroxide added in 20 minutes.

TABLE 2

SPECIFICITY OF THE CHOLINESTERASE OF THE HEADS OF HOUSE FLIES

| Substrate 0.045 M | Cubic Millimeters of Carbon Dioxide Evolved in 15 Minutes |
|---|---|
| Acetylcholine bromide | 132 |
| Acetyl-β-methylcholine bromide | 64 |
| Benzoylcholine bromide | 9 |

### THE EFFECT OF SUBSTRATE CONCENTRATION

The effect of varying the substrate concentrations on enzyme activity was determined for the range of 0.5–0.00001 M, and the rate (cubic millimeters of carbon dioxide evolved in 15 minutes) plotted against *pS*, the negative log of the molar concentration. That the activity is inhibited by high substrate concentration is shown in Figure 1. The dissociation constant of the enzyme-substrate complex, *Ks*, calculated by the method of Lineweaver and Burk (1934) was $1 \times 10^{-3}$.

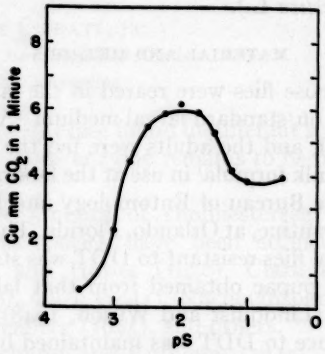


FIG. 1.—The effect of substrate concentration (*pS*) on the cholinesterase activity of the heads of house flies.

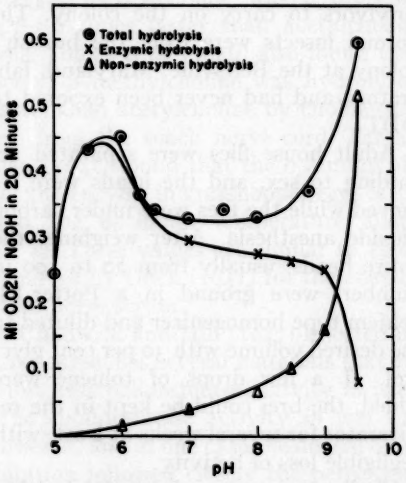


FIG. 2.—The effect of pH on the cholinesterase activity of the heads of house flies.

### THE EFFECT OF pH

The titration method was used to determine the effect of pH on the hydrolysis of acetylcholine bromide. The results are shown in Figure 2.

## DISCUSSION

Only one report of differences in cholinesterase activity due to sex has been noted in the literature. Caridroit, Kaswin, and Serfati (1948) found greater enzymatic activity in the plasma of the rooster than in that of the hen. The activity in capons was greatly reduced but was restored by testosterone propionate. In human plasma, Vahlquist (1935) showed no difference due to sex.

Although strains of various species of insects have been described as differing from "normal" strains in their resistance to insecticides (Babers, 1949), few other physiological differences among the strains have been noted. Hardman and Craig (1941) observed that California red scale (*Aonidiella auranti* [Mask.]) which were resistant to fumigation with hydrocyanic acid immediately closed their spiracles on exposure to the gas and kept them closed about 30 minutes. The nonresistant, or normal, scales could keep the spiracles closed only 1 minute, and death rapidly ensued. Gough (1939) reported that confused flour beetles (*Tribolium confusum* Duv.) resistant to fumigation with hydrocyanic acid used 2.803 cu. mm. of oxygen/mg/hr, while the normal insect only used 2.163 cu mm/mg/hr.

The difference in cholinesterase activity between the normal and the DDT-resistant house flies is then of considerable interest. The difference in activity at various age levels is also of interest. No explanation for these differences is offered at this time. This experiment was replicated three times, and different generations of flies were used for each replication. Only the results of using the titration method are given in Table 1, but similar results were obtained by the Warburg method. The enzyme from both resistant and nonresistant strains had similar properties, so far as has been determined.

The pattern of specificity of the enzyme as shown in Table 2 is different from that reported by Richards and Cutkomp (1945) for the cholinesterase of the honeybee brain and the cockroach nerve cord. As mentioned above, these workers found that acetyl-$\beta$-methylcholine was hydrolyzed 2.7–3.0 times as fast as acetylcholine.

The inhibition of cholinesterase activity by excess substrate indicated by the bell-shaped curve in Figure 1 is not unique for the enzyme obtained from the heads of house flies. In a review by Bodansky (1946) on cholinesterase, the theory of this type of inhibition is discussed, and reference is made to several authors who noted the phenomenon in the enzyme from various species other than insects. Augustinsson and Nachmansohn (1949) bring out the importance of determining the effect of substrate concentration when attempts are being made to describe an enzyme.

The observed maximum activity occurs when the concentration of acetylcholine is about 0.01 M. Up to this point the acetylcholine esterase in fly heads is similar to that in the tissues of several other species of animals. However, our observations on the effect of pH on activity are at considerable variance with those reported for the enzymes obtained from other sources. Glick (1941) reported 8.5 as the optimum pH for the enzyme from horse serum, and Tahmisian (1943) found the same value for the enzyme obtained from developing grasshopper eggs. Although the optimum pH of the cholinesterase of fly heads was found to be about 5.75, when correction was made for non-enzymic hydrolysis, there was little rate change from pH 6.25 to pH 9.00. This activity over such a wide pH range not only

was unexpected but is also unexplained. Small changes in substrate concentration had much greater effect than did large pH variation. The titration method only was used in this determination, since the Warburg technique cannot readily be employed over such a wide pH range without changing buffers.

## SUMMARY

The cholinesterase activity in the heads of house flies (*M. domestica* L.) resistant to DDT was lower during the first 5 days after emergence than was the activity in the normal fly. The male flies, on a weight basis, in both resistant and normal colonies, showed more cholinesterase activity than did the females. The activity varied from day to day, but no regular pattern was observed. Acetylcholine was hydrolyzed about twice as fast as was acetyl-β-methylcholine and about seven times as fast as benzoylcholine. The reaction was inhibited by excess substrate, and the optimum pH for the enzyme was about 5.75. Little rate change was noted between pH 6.25 and pH 9.0.

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# THE COMBINED ACTION OF 2,4-DINITROPHENOL AND ETHYL CARBAMATE (URETHANE) ON THE OXYGEN UPTAKE OF EMBRYONIC CELLS[1]

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THAT 2,4-dinitrophenol (D.N.P.) and other substituted phenols have marked accelerating effects upon the oxygen consumption of various tissues and cells seems well established (Bodine and Boell, 1938; Clifton, 1946). The mode of action of these compounds upon the cellular respiratory mechanisms, however, does not seem to be so well understood, since numerous variations or differences in interpretation of data seem to exist. Clifton (1946), in a rather critical review, points out that, for many organisms, oxygen consumption is greatly accelerated, while assimilation or syntheses are markedly inhibited. He also indicates that D.N.P. may affect the oxygen consumption of cells in the presence of different and similar substrates in a varied manner not only for different species but also for organisms within the same species. Lardy, Ghosh, and Plaut (1949), in working with the metabolic activity of mammalian sperm, compare the activity of D.N.P. with that of an inherent biological regulator, in that it stimulates cellular respiration and aerobic glycolysis, while preventing energy utilization. For sea-urchin ova, Krahl and Clowes (1940) have pointed out the possible relationships between the respiratory effects and the division-inhibiting effects of the substituted phenols. Rather clear-cut evidence exists that D.N.P. not only stimulates oxygen uptake of cells but also has a marked inhibitory effect upon phosphate metabolism (Hotchkiss, 1943). Details as to the manner in which these effects are produced are still obscure. The present results are based upon an investigation of the combined effects of D.N.P. and ethyl carbamate (urethane) (E.C.) on the oxygen uptake of the cells of the embryo of the grasshopper, *Melanoplus differentialis*.

[1] Aided by grant from the National Institute of Health. Acknowledgment is gratefully made to Laurence R. Fitzgerald, Etta Andrews, John Johnston, and Herman Tharp for technical assistance in carrying out these experiments.

## MATERIAL AND METHODS

Embryos, free from yolk, were dissected from eggs of the grasshopper as previously described (Bodine and Fitzgerald, 1948). One hundred embryos of known age were employed in each respiration flask (capacity 5 ml.), and oxygen determinations were carried out by means of standard Warburg manometers at 25° C. Both mitotically active and blocked embryos were employed; but, since results are essentially similar, all data herein reported are for mitotically active ones. Ringer solution (pH 6.8) was

used as the suspension medium, and all chemicals employed were made up in it. The concentrations of D.N.P. and E.C. given are final ones in the respiration flasks, due consideration being given to concentrations in side arms before mixing or pouring contents into the flasks.

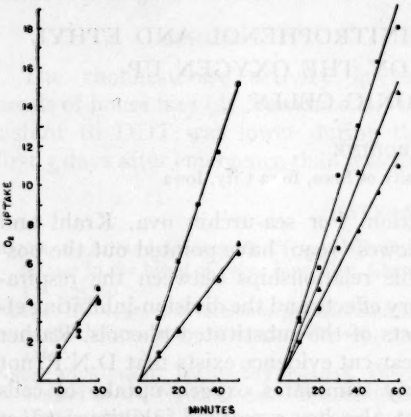


FIG. 1.—Effect of 0.1 M E.C. and $1 \times 10^{-5}$ M D.N.P. on the oxygen uptake of embryos. *Ordinate*, $O_2$ uptake in cubic millimeters per 100 embryos: *abscissae*, time in minutes. Divisions of abscissae represent: (1) control period in Ringer, *extreme left;* (2) *middle*, effect of contents of first side arm; (3) *exrteme right*, effects of contents of second side arm. Experiment ran continuously, and zero (*o*) minutes on abscissae represent time at which contents of side arms were emptied into respiration flask. Total duration of experiment equals sum of time shown on abscissae. Concentrations of all reagents in respiration flask were maintained at constant levels by controlling concentrations of same added from side arms. All concentrations given represent final ones in respiration flasks. *Open circles*, Ringer (control); *squares*, first side arm, $1 \times 10^{-5}$ M D.N.P.; second side arm, 0.1 M E.C.; *triangles*, first side arm, 0.1 M E.C.; second side arm, $1 \times 10^{-5}$ M D.N.P.

bryos to the reagents. Checks without embryos were run for all experiments.

## RESULTS

Previous investigations have shown that D.N.P. produces optimal reversible stimulation of oxygen uptake of grass-

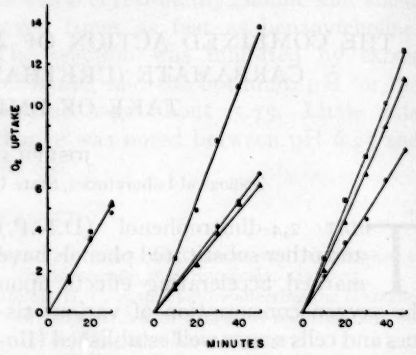


FIG. 2.—Same as Fig. 1 except for 0.2 M E.C.

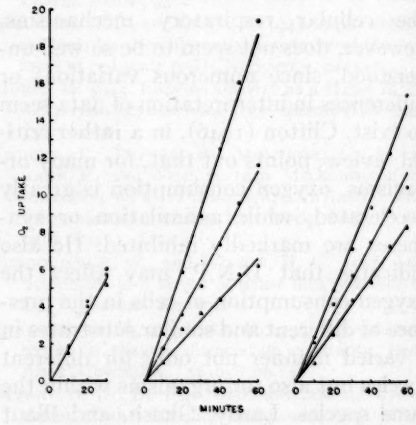


FIG. 3.—Same as Fig. 1 except for 0.3 M E.C.

Controls for all experiments were run in Ringer solution for periods of 30–60 minutes, after which the contents of the side arms were poured into flasks. Exposures to reagents were for 50–60 minutes. Chemically pure D.N.P. and E.C. were employed, and particular attention was paid to the sequence of exposure of em-

hopper embryos at concentrations of approximately $1 \times 10^{-5}$ M (Bodine and Fitzgerald, 1949). This concentration has therefore been employed throughout the present experiments. E.C. produces stimulation of oxygen uptake in low doses and reversible decreases in higher concentrations (Bodine and Fitzgerald,

1948). Combinations of D.N.P. ($1 \times 10^{-5}$ M) and E.C., ranging in concentration from 0.1 to 1.0 M, have been used. Typical results of experiments are graphically represented in Figures 1–8.

Embryos, treated with low concentrations of E.C. (0.1–0.2 M), show little if any marked changes in oxygen uptake (Figs. 1 and 2). When they were subsequently exposed to D.N.P. ($1 \times 10^{-5}$ M), however, marked increases in the oxygen uptake occurred and closely resembled those found for embryos treated with D.N.P. alone (Figs. 1 and 2). As the concentration of E.C. is increased and marked reversible reductions (60–75 per cent) in the oxygen uptake occur, subsequent additions of D.N.P. no longer produce stimulating effects (Figs. 3–7), and responses of embryos are then quite sim-

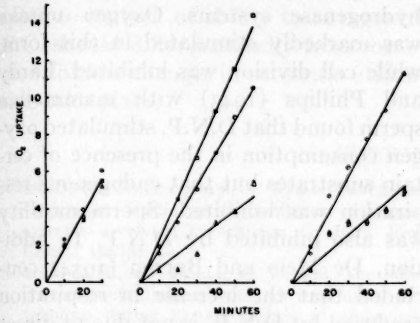


FIG. 4.—Same as Fig. 1 except for 0.4 M E.C.

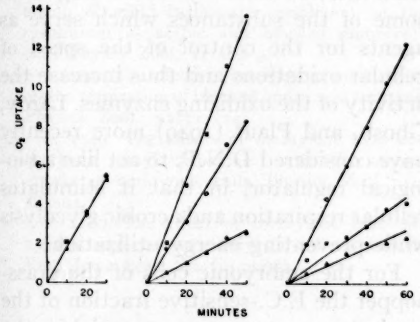


FIG. 5.—Same as Fig. 1 except for 0.5 M E.C.

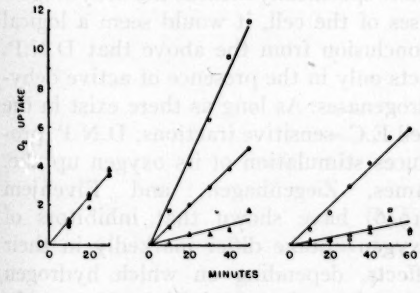


FIG. 6.—Same as Fig. 1 except for 1.0 M E.C.

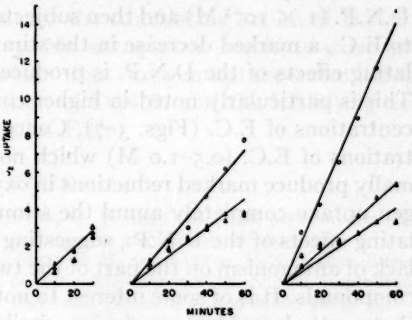


FIG. 7.—Same as Fig. 1 except for 0.4 M E.C. Solid triangles equal E.C. throughout.

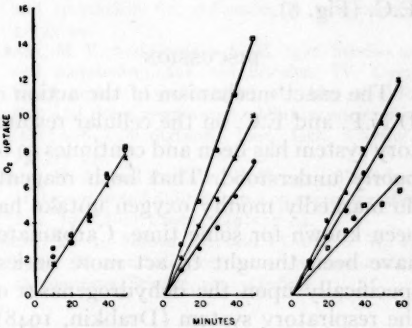


FIG. 8.—Effect of 0.4 M E.C. and $1 \times 10^{-5}$ M D.N.P. and $3 \times 10^{-5}$ M methylene blue on the oxygen uptake of embryos. Same as Fig. 1 except that crosses represent methylene blue ($X$ = first side arm, $3 \times 10^{-5}$ M M.B.; second side arm, 0.4 M E.C.). Note marked differences in response to E.C. by D.N.P. (*squares*) and methylene blue (*crosses*).

ilar to those treated with E.C. alone. Prior treatment with E.C. seems to retard slightly the stimulation due to subsequent addition of D.N.P., and responses are not so marked as when D.N.P. treatment precedes that of E.C.

If embryos are first treated with D.N.P. ($1 \times 10^{-5}$ M) and then subjected to E.C., a marked decrease in the stimulating effects of the D.N.P. is produced. This is particularly noted in higher concentrations of E.C. (Figs. 3–7). Concentrations of E.C. (0.5–1.0 M) which normally produce marked reductions in oxygen uptake completely annul the stimulating effects of the D.N.P., suggesting a lack of antagonism on the part of the two compounds. It is of some interest to note that methylene blue, producing similar stimulating effects to the D.N.P., is not affected by the subsequent addition of E.C. (Fig. 8).

## DISCUSSION

The exact mechanism of the action of D.N.P. and E.C. on the cellular respiratory system has been and continues to be poorly understood. That both reagents do markedly modify oxygen uptake has been known for some time. Carbamates have been thought to act more or less specifically upon the dehydrogenases of the respiratory system (Drabkin, 1948). Within reversible limits it can be shown that approximately 25–30 per cent of the entire respiratory system in the grasshopper embryo is carbamate-insensitive, while the remainder is quite susceptible to the reagent (Bodine and Fitzgerald, 1949). The D.N.P.-stimulated fraction is also quite susceptible to the action of E.C. and other compounds (CO, cyanide) (Clifton, 1946). Since D.N.P., carbamates, and other reagents produce marked changes in the respiratory quotient (Bodine and Fitzgerald, 1949), it leads one to suggest that substrate-enzyme relations must be influenced by such drugs. For microörganisms Clifton (1946) has shown that the action of D.N.P. may vary with the nature of the substrate and of the organism. In the case of the sea-urchin egg Krahl and Clowes (1940) conclude that the substituted phenols have no effect upon the dehydrogenase systems. Oxygen uptake was markedly stimulated in this form, while cell division was inhibited. Lardy and Phillips (1943) with mammalian sperm found that D.N.P. stimulated oxygen consumption in the presence of certain substrates but that endogenous respiration was inhibited. Sperm motility was also inhibited by D.N.P. In addition, De Meio and Barron (1934) concluded that the increase in respiration produced by D.N.P. is not due to direct oxidation of the oxidizable substrates but that D.N.P. acts by combining with some of the substances which serve as agents for the control of the speed of cellular oxidations and thus increase the activity of the oxidizing enzymes. Lardy, Ghosh, and Plaut (1949) more recently have considered D.N.P. to act like a biological regulator, in that it stimulates cellular respiration and aerobic glycolysis while preventing energy utilization.

For the embryonic cells of the grasshopper the E.C.-sensitive fraction of the respiratory system seems essential for the stimulating action of D.N.P. If E.C. more specifically attacks the dehydrogenases of the cell, it would seem a logical conclusion from the above that D.N.P. acts only in the presence of active dehydrogenases. As long as there exist in the cell E.C.-sensitive fractions, D.N.P. produces stimulation of its oxygen uptake. Ames, Ziegenhagen, and Elvehjem (1946) have shown that inhibitors of oxygen uptake differ markedly in their effects, depending on which hydrogen donor is used as the initial substrate. In

the case of the grasshopper embryo the normal R.Q. (0.75) is markedly increased (0.95) under the influence of D.N.P., as well as of E.C. (Bodine and Fitzgerald, 1949). Methylene blue, on the other hand, has little if any effect on the R.Q. value. It seems reasonable, therefore, to assume that the action of D.N.P. in respect to its lack of ability to antagonize E.C. should differ markedly from methylene blue if changes in substrate utilization take place.

## CONCLUSIONS

1. The combined effects of D.N.P. and E.C. upon the oxygen uptake of embryos of the grasshopper, *M. differentialis* have been studied.

2. E.C.-sensitive fractions of the cellular respiratory mechanism seem essential for the stimulating action of D.N.P.

3. No antagonism seems to exist between the oxygen-uptake–stimulating action of D.N.P. and the inhibiting action of E.C.

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# NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

## *PHILOSOPHY FOR THE FUTURE: THE QUEST OF MODERN MATERIALISM*
## EDITED BY ROY WOOD SELLARS, V. G. McGILL, AND MARVIN FARBER[1]

*Reviewed by* RALPH S. LILLIE
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THIS volume is a symposium on problems of philosophy and has a special interest for biologists because of its prevailingly naturalistic temper and scientific point of view. The topics are highly varied; many contributors are concerned with traditional problems, but some papers are purely scientific; others are historical or social; and there is even one on economics. They are incompletely unified, except that all authors seem agreed on the importance of extending scientific method in philosophy as far as possible. The practical note is sounded in many places; there is much discussion of values, ethical problems, and problems of social reform. An Introduction signed by the editors explains the purpose and occasion of this co-operative enterprise. The chief aim is to put modern philosophical knowledge on as firm a foundation as possible; but a humanistic faith and motive are also acknowledged: "Modern materialism is opposed to any other criterion of human value and policy than human needs and aspirations" (p. ix). There is, of course, nothing antireligious about the book, but also little that is religious as such.

Several biologists have contributed papers, including C. J. Herrick J. B. S. Haldane, T. C. Schneirla, and (if we regard psychoanalysts as biologists) Judd Marmor; and the interpretations and outlook of many other contributors are essentially biological; this is true, for example, of the papers by V. J. McGill ("A Psychological Approach to Personality"), J. R. Reed ("The Nature and Status of Values"), and L. A. White ("Ethnological Theory"). The essay by G. P. Conger (one of the best in the book), entitled "Pragmatism and the Physical World," shows the close relation between pragmatism and biological conceptions. We may summarize the prevailing point of view briefly as follows. The living organism is regarded as a natural system, interacting self-preservatively or adaptively with an environment; this system is psychophysical; its reactions are both physiological and psychological; knowledge has its neurophysiological correlate and foundation. As C. I. Lewis expresses it: "The active attitudes of animal life presumably represent the phenomena from which genetically the human type of knowing has arisen" (quoted on p. 538). Similarly, human values are regarded as having a biological foundation.

Throughout the book the treatment and emphasis are essentially scientific. Human experience has its constant and verifiable factors and categories; under the term "experience" is to be understood experience of the external world as gained through the senses as well as purely inner experience. But experience is not nature itself; it is not a complete but a partial index of natural reality, essentially selective and referential in character; lying behind it as background is an extra-experiential field of reality which is vastly more extensive than any human experience can possibly be. Positivism is deprecated (see the papers of M. Cornforth, "Logical Empiricism," and E. J. Nelson, "The Category of Substance"); experience is not self-sustaining but is the imperfect reflection of an independent reality or cosmos whose characters are best determined by scientific investigation. Such a view implies rejection of the purely idealistic view of nature as represented in the line of philosophers from Berkeley to Husserl; as one of the editors, Marvin Farber, puts it (p. 594), "Experience is in nature, and not nature in experience," i.e.,

[1] New York: Macmillan Co., 1949. Pp. xiv+657. $7.50.

nature has priority over experience. While this may be an unproved hypothesis, at least it is one which is confirmed overwhelmingly at all points, and no more can be said for any scientific hypothesis. To say this is not inconsistent with acknowledging the priority of experience in a certain sense, expressed neatly by Professor Conger: "We are all in the experiocentric predicament" (p. 523). But it is impossible to regard experience as prior in the genetic or cosmological sense; all the evidence indicates that man, with his special material organization and spiritual endowment, has evolved by a gradual process within an independent physical nature, unlimited in its complexity, spatial extent, and duration. The essay by Roy K. Marshall ("An Astronomer's View of the Universe") gives an impressive account of the conclusions reached by modern astronomy regarding the extent and physical structure of the cosmos. There are other papers by physicists: the subject of relativity is reviewed briefly by L. Infeld ("On the Structure of Our Universe"), and quantum theory by Melba Phillips ("Quantum Mechanics"); an interesting paper by J. B. S. Haldane ("Interaction of Physics, Chemistry, and Biology") considers some of the possible modes of transition from nonliving physical nature to living organisms.

Apparently, most authors believe that future progress in philosophy must be based on the positive results and conclusions of science rather than on conceptions evolved speculatively or developed on a basis of a priori reasoning. Logic, while indispensible, is to be regarded as having a natural foundation—i.e., as being a formalized statement of the constant categories and modes of connection which interpenetrate all nature and experience. The essay by Dirk J. Struik ("Mathematics") illustrates this view, of which the best known modern exponents are Bertrand Russell and John Dewey. Repetition, order, sameness, uniformity, classifiability, are facts of nature, idealized and rationalized in the logical systems. The subtitle, "The Quest of Modern Materialism," may seem strange to many readers; the term "materialism," however, is not to be interpreted in the popular (much less "crass") sense but rather as having reference to the modern physical view of the world, according to which localized, spatially extended, and atomically subdivided material and energy constitute the structural foundation of the natural process. The word does not imply that the external or objective (physical) rather than the subjective (psychical) aspects of nature alone have true reality. Such a distinction can no longer be regarded as final; priority cannot be assigned to either "mind" or "matter"; the separation of mind and body as two essentially different things or agencies which interact is to be regarded as scientifically unwarranted and derived from the uncritical use of a "thing-language" (cf. the paper by Wilfrid Sellars entitled "Aristotelian Philosophies of Mind," e.g., p. 569). The emphasis on the "material" is rather an emphasis on what is permanent and established in nature; this natural permanency exhibits both conservation (e.g., of energy) and a cumulative historical background of change and development leading up to the present. The "mental" appears transient and unsubstantial by contrast. In general, the material is more amenable to scientific treatment than the mental: experimentally we find matter to be characteristically regular and constant in its behavior, while mind is largely unpredictable.

This experimental constancy of the physical implies a foundation of ontological constancy, i.e., a stable, substantial basis or matrix (analogous to Aristotle's "unmoved mover") which underlies all phenomena. We have here the problem of "substance," considered critically in the paper by Everett J. Nelson ("The Category of Substance"). Substance is that field of reality which is antecedent to all special historical becoming and evolution; its constancy provides the ground for the ontological synthetic connections of nature (p. 123). Substance corresponds to permanent potentiality or predisposition (cf. also Wilfrid Sellars on universals. pp. 546 ff.); its stability (of which natural law is one expression) is reflected in the stable properties and routine activities of physical nature. But it must be remembered that the scientific concepts of matter and energy are abstract and correspondingly limited in their applicability. To refer all natural activity to the automatic and unguided play of atomic units and energies is an unwarranted form of "reductionism." Many levels of integrated structure and activity are found in nature, each with its characteristic laws or constants. Each level has its own emergent properties which cannot be entirely described in terms of lower levels; all levels, including the social, have their special constants and their special modes of measurement, not necessarily reducible to physical measurement (cf. C. West Churchman, "A Materialist Theory of Measurement," pp. 481 ff.). Accordingly, the answer to the question put by Haldane (p. 202) —"Can we give an account of the phenomena of

life in terms of physics and chemistry?"—while affirmative in one sense, must be negative in another; i.e., such an account already exists, but it is incomplete and must remain so; the phenomena of one level are only partly reducible to those of a lower level (for levels cf. especially C. J. Herrick, "A Biological Survey of Integrative Levels," pp. 222 ff.; also T. C. Schneirla, "Levels in the Psychological Capacities of Animals," pp. 243 ff., and Bernhard Stern, "Some Aspects of Historical Materialism," pp. 340 ff.). "Modern empirical naturalism is free from puppetry or reductionism" (R. W. Sellars, "Materialism and Human Knowing," p. 104).

Close observation shows a certain lack of finality or complete determinateness in all single events; hence scientific constants have to be determined statistically, as approximations. Limits to finality are set by the individual uniqueness of each separate entity or event. The universe is not a closed system; the openness and incompleteness of nature and experience must be recognized in any realistic philosophy (cf. especially M. Farber, "Experience and Subjectivism," pp. 592 ff.). The natural advance into the future always contains some element of novelty and unpredictability. This unpredictability has its origin in the individual autonomy and increases with complexity of organization: there is an internal directedness which increases with life (R. W. Sellars, "Materialism and Human Knowing," p. 104). Here we have the recognition of a teleological factor in nature, finding its expression especially in the higher organisms; but not all authors share this view. T. C. Schneirla ("Capacities of Animals") regards the belief in a generalized teleology in living organisms as outmoded and fallacious, and refers all behavior to purely physiological factors. Bernhard Stern, however ("Historical Materialism," pp. 341 ff.), warns against an exaggerated emphasis on external and material factors in history and quotes with approval R. R. Marett (*Psychology and Folklore* [London, 1920]): "Human history is no Madame Tussaud's show of decorated dummies. It is instinct with purposive movement through and through." But, apart from Schneirla's paper, the book contains little discussion of purpose and teleology. Human behavior is apparently for the most part regarded as determined by historical factors and external environment; this is illustrated in the papers by V. J. McGill ("A Psychological Approach to Personality") and Judd Marmor ("Psychoanalysis"). Incidentally, it may be noted that in both these papers the importance of the genetic biological factor—i.e., of purely hereditary characteristics as determined by the special constitution of the nuclear gene-complex—seems to be neglected or underestimated, although this is perhaps the chief factor in the determination of personal differences.

The emphasis throughout the book on the constant and regular features of experience—i.e., those representable more or less adequately by language, mathematics, and other symbolisms—is greater than might have been expected in a philosophical work; a definite tendency or bias in modern thought is here reflected. The novel, creative, and unformulable features of natural activity receive comparatively little attention (see, however, R. W. Sellars, "Materialism and Human Knowing," p. 103); there is little reference to Bergson and Whitehead and none to G. H. Mead; the word "future" is not in the Index. This is perhaps not surprising; facts (*facta*), the material of science, belong in the past and present; the future lacks the certainty which is the special aim of science. This uncertainty, based, as it appears to be, on the individualizing activity of nature, is not entirely avoided by measurement and statistical methods. It is true that a large part of natural activity is predictable; and a recognized criterion of science is its ability to predict. Prediction is extrapolation based on uniformities found in the past, but often the question is: How far can these be relied upon for the future? The unpredictable features of individual activity reach their climax in the incalculable decisions of human beings; what happens in the human sphere depends more upon these arbitrary individual events than upon any scientifically determinable social laws. Decisions are made in the immediate present; accordingly, it would seem that the more specifically philosophical problems must be those which are concerned primarily with immediacy and the charactistics of the temporal present—that "concrete slab of nature" (to use Whitehead's phrase) in which "something is going on"—rather than with the past—the repository of established fact which is the special concern of science. Sellars, Conger, and Farber reccognize this; but, on the whole, the problem of novelty is insufficiently considered. Most of the contributors, however, indicate a faith in progress. Farber, although phenomenologist and expositor of Husserl, deplores the latter's hyperidealistic ontology and adopts a position which is essentially naturalistic, emphasizing especially the possibilities of future evolution. He (as well as others) urges thinking men to recognize their

responsibility for improving the general conditions of human life. It is noteworthy that the social ideas of Karl Marx attract the interest of many contributors; Marx, in fact, has as large a place in the Index as any other author, not excluding Kant or Aristotle.

There are many other features of this interesting book that deserve extended comment, but space is limited, and I have confined myself chiefly to those which are of more special interest to biologists. I may add that in recognizing the artificiality of the distinction between science and philosophy the book represents a return to the point of view of Aristotle.

## *RADIOACTIVE INDICATORS: THEIR APPLICATION IN BIOCHEMISTRY, ANIMAL PHYSIOLOGY, AND PATHOLOGY*, BY GEORGE HEVESY[1]

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THE use of radioactive tracers in the study of biological processes has increased by many times during the last ten years, if one can judge from number of published papers and review books. The present volume summarizes a large portion of the biological experiments with tracers published prior to 1948. The author, George Hevesy, is the leader of the Scandinavian school of tracer biology, and much Scandinavian research is here described, along with work published in other countries, particularly the United States. This book deals with the use of radioactive tracers in animals, and a later volume is promised, dealing with tracers in research on plants.

Tracer methods provide the most useful approach to the dynamic relations in biochemistry, turnover of elements, metabolic rates, and similar functions; and radioactive tracers are more easily used than heavy tracers, which require a mass spectrograph. However, tracer methods and results per se cannot become an independent science, and the results described in this book are drawn from diverse fields, such as salt balance, cell permeability, intermediary metabolism, nucleic acid metabolism, and circulatory physiology. It is unlikely that a revision of this book will ever be published, but rather the material treated here will probably be incorporated into the various subject-matter areas. The inherent difficulty of writing a book about the results of a single technique is apparent, since some data are presented in chapters dealing with specific elements, others under certain tissues, and still other similar data in chapters on certain physiological characteristics. Thus, for example, material on red blood cells is scattered in many parts of the book. The twenty-nine-page subject index is, however, excellent, and, by using it, specialists in many fields will be able to locate, in the text, summaries of material of their particular interests.

The first five chapters (94 pp.) deal with the production and measurement of radioactive isotopes. Discussion of physical theory is brief—in fact, too abbreviated to be of much use in providing a background for biologists; also the discussion of counting techniques is limited. A Segri chart of the more important properties of both stable and radioactive isotopes is included, also an Atomic Energy Commission price list as of September, 1947. The description of methods of chemical separation of different elements for determination of specific activity (radioactivity per unit weight of an element )is a very useful section. Throughout the book emphasis is given to the radioactivity of specific chemical fractions rather than merely as percentage of total administered radioactive material.

Chapter vi (94 pp.) summarizes data on the absorption, distribution, and excretion of various elements. This includes the early tracer work on lead (isotopic with radium D); on elements of physiological importance, such as phosphorous, iodine, iron; and on minor elements, including some fission products and heavy metals. The next three chapters (218 pp.) deal with permeability, turnover of various organic fractions, and intermediary metabolism. Detailed data are presented, fully documented and well tabulated. Phosphorus has been used more than any other tracer element; it is readily available, easily measured, and combines in many organic compounds. Perhaps the most important generalization emerging from tracer studies is the concept of rapid flux or two-way movement of many elements across cell membranes. Cells such as red blood corpuscles, which by chemical methods appear to be relatively impermeable to cations, are now shown to

[1] New York: Interscience Publishers, 1948. Pp. 556+97 figs. $10.00.

have a relatively high exchange rate for sodium and potassium. The incorporation of phosphate into organic compounds, once it is inside many cells, is almost immediate. The rate of turnover of different substances differs; for example, molecules of ribonucleic acid are replaced many times more readily than those of desoxyribonucleic acid. Tracer methods also demonstrate the direct transfer of phosphate from one intermediate to another in carbohydrate metabolism. Tracer methods have not yet contributed much to our knowledge of the molecular structure of cell membranes or the form in which substances penetrate membranes.

Chapters x and xi (99 pp.) deal with tracer experiments on turnover in skeletal material and red blood corpuscles. The rate of utilization of calcium and phosphate is high in growing bone. There is also some replacement in fully calcified bone and teeth. Tracers have been used for study not only of permeability and metabolism of red blood cells but also of their maturation, length of life, and blood volume. Applications in blood diseases are mentioned.

The final chapter considers the two assumptions always made in work with radioactive tracers: (1) that it makes little difference biochemically which isotope of an element is presented to a cell and (2) that the amount of ionization produced by radioactive tracers is negligible. Each of these assumptions is valid within limits which must be recgonized for dependable tracer research.

Hevesy's summaries of experimental data are well written, but one wishes that each section might end with a sentence or two of general conclusions; too frequently, data are presented without interpretation. However, since the data are drawn from many fields, this book will be a valuable source book and will be regularly consulted by physiologists, biochemists, and investigators in experimental medicine.